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**MANIPULATIONS OF THE REPRODUCTIVE PHYSIOLOGY AND  
ENDOCRINOLOGY OF THE EWE**

Corpus Luteum function after the induction of oestrus  
in two periods of the breeding season, termination of  
early pregnancy with prostaglandins and the effects of  
PGE2 administered at dioestrus in the cervix and  
vagina.

**BY**

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## **LIST OF CONTENTS**

<b>ACKNOWLEDGEMENTS</b>	<b>i</b>
<b>ABSTRACT</b>	<b>ii</b>
<b>AUTHOR'S DECLARATION</b>	<b>iv</b>
<b>CHAPTER 1</b>	<b>1</b>
<b>1.1 GENERAL MATERIAL AND METHODS</b>	<b>1</b>
1.1.1 Experimental design	1
1.1.2 Animals studied	2
1.1.3 Habitat and management of the animals	2
1.1.4 Synchronization of the oestrous cycle	3
1.1.5 Matings	4
1.1.6 Oestrus detection	4
1.1.7 Blood sampling	5
1.1.8 Quantification of Progesterone: (ELISA assay)	5
<b>1.2 CONTROL EXPERIMENT STUDYING SYNCHRONIZATION USING PROGESTAGEN SPONGES</b>	<b>6</b>
1.2.1 Introduction	6
1.2.2 Design, Material and Methods	8
1.2.3 Results	10
1.2.4 Discussion	13
<b>1.3 STUDIES ON LUTEAL FUNCTION DURING DIFFERENT PARTS OF THE BREEDING SEASON</b>	<b>19</b>
1.3.1 Introduction	19
1.3.2. Material & Methods	26
1.3.2.1 Ewes	26
1.3.2.2 Synchronization and superovulation	26
1.3.2.3 Matings	27
1.3.2.4 Monitoring of the cycle	27
1.3.2.5 Hormone Assays	28
1.3.3 Results	29
1.3.4 Discussion	36
<b>CHAPTER 2</b>	<b>48</b>
<b>2.1 INTRODUCTION</b>	<b>48</b>
<b>2.2 MATERIAL AND METHODS</b>	<b>51</b>
2.2.1 Experiment 1: Lutalyse administered at different times of the pregnancy	51

2.2.2	Experiment 2: comparison between Lutalyse and Estrumate	52
2.2.3	Blood Sampling and Progesterone Determination	52
2.2.4	Repetition of Treatments	52
2.2.5	Slaughterhouse material	56
<b>2.3</b>	<b>RESULTS</b>	<b>56</b>
2.3.1	Classification of the responses to the treatment with prostaglandins	56
2.3.2	Resistance to PGF2a (Lutalyse)	58
2.3.3	Estrumate versus Lutalyse	60
2.3.4	Estrumate versus Lutalyse: speed of the Luteolysis	61
2.3.5	Post-mortem findings	61
<b>2.4</b>	<b>DISCUSSION</b>	<b>67</b>
2.4.1	The pattern of the effect of Lutalyse	67
2.4.2	Comparison of Estrumate with Lutalyse	72
2.4.3	Dose	74
2.4.4	The effect of second administration of prostaglandins	77
<b>CHAPTER 3</b>		<b>81</b>
<b>3.1</b>	<b>INTRODUCTION</b>	<b>81</b>
<b>3.2</b>	<b>MATERIAL AND METHODS</b>	<b>89</b>
3.2.1	Experiment 1	89
3.2.2	Experiment 2	90
3.2.3	Microscopy processing	91
3.2.3.1	Light Microscopy (LM)	91
3.2.3.2	Scanning Electron Microscopy	91
<b>3.3</b>	<b>RESULTS</b>	<b>92</b>
3.3.1	Experiment 1	92
3.3.1.1	Pessary retention	92
3.3.1.2	Passage of the Cervix after the treatment with PGE2 and E2	92
3.3.2.	Experiment 2	95
3.3.2.1	Ultrastructural studies	95
<b>3.4</b>	<b>DISCUSSION</b>	<b>125</b>
<b>REFERENCES</b>		<b>138</b>

## ABSTRACT

The study is divided into three main parts, which investigate the manipulation of reproduction in the ewe with various drugs. The first part investigates the luteal function during and following progestagen synchronization of oestrus. In the second part, the efficacy of two commercially available prostaglandins (PGs) in inducing luteolysis and termination of early pregnancy is assessed. The third part studies the effect of intravaginally administered prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) on the cervix and vagina during the luteal phase.

In the first part, the lifespan of the CL (assessed by daily progesterone levels and oestrous detection) was investigated during two periods of the breeding season following oestrus induction with progestagens alone (controls) or with a superovulatory dose of PMSG (PMSG+). Short cycles (<12 days) were observed at the peak of the season and in association with the use of PMSG. Results suggested that insufficiency of gonadotrophins to support the CL was not the mechanism involved in the premature CL demise. The CL function was also studied during the progestagen treatment (MPA) which was administered on different known days of the cycle. MPA, when administered at metoestrus did not suppress the maintenance of the existing CL in most ewes (6/8). This result indicates that if the CL outlasts the lifespan of the exogenous progestagen, the resulting asynchrony may be mistaken for premature regression of the supposedly induced CL.

There are conflicting results about the time after maternal recognition when the CL recovers its responsiveness to PGs. Progesterone was measured (with ELISA) following PG injections at various stages of early pregnancy at doses which are luteolytic in cyclic ewes.

On day 20 of pregnancy resistance was observed towards both PGs, however Estrumate produced faster luteolysis and a higher frequency of abortions than Lutalyse. Resistance to Lutalyse was demonstrated on day 28 of pregnancy, which encourages more studies in the maintenance of pregnancy after maternal recognition and embryonic attachment.

In the third part, the ripening effect of intravaginal PGE<sub>2</sub> alone or in combination with oestradiol in early pregnancy or at oestrus was evaluated. Treatment with PGE<sub>2</sub> produced softening of the cervix but did not allow complete catheterisation. Further studies were carried out on superovulated ewes on day 6 of the cycle (PGE<sub>2</sub> treated vs controls), using histology and SEM of the vagina and three portions of the cervix. The ultrastructural changes observed in the CT of both the vagina and cervix of the treated animals were similar to those described in sheep and humans undergoing physiological ripening of the cervix at pregnancy and parturition. Changes were greater at the external os, suggesting that PGE<sub>2</sub> administered intracervically may be advantageous. Squamous metaplasia was also observed in treated ewes, a change which has neither been described in the ewe nor man and warrants further investigation as metaplasia is a precancerous lesion in humans.



## DECLARATION

I, Lina Audicana, do hereby declare that the work presented in this thesis is original, was carried out by me and has not been presented for an award of a degree in any other University.

Date: April 1992

## **Chapter 1**

### **STUDIES ON OESTROUS INDUCTION AND SUBSEQUENT LUTEAL FUNCTION IN SHEEP**

#### **1.1 GENERAL MATERIAL AND METHODS**

##### **1.1.1 Experimental design**

Oestrus and superovulation were induced on three occasions during the breeding season of 1989-90, in November, February and March. In February, oestrus without superovulation was also induced in a number of ewes, splitting the flock into two groups: superovulated (PMSG+) and non-superovulated sheep (controls).

The induction of oestrus enabled 2 experiments to be carried out. The first (described in section 1.3) consisted of monitoring oestrous behaviour and progesterone profiles during the induced cycles to investigate the possible incidence of short cycles (<12 days long) both in the peak of the breeding season (November) and also late in the breeding season (February and March).

In the second, pregnant ewes resulting from the matings at the induced oestruses were treated with prostaglandins (PG) and the resulting luteolysis monitored using plasma progesterone concentrations. Preliminary work included treatment of superovulated ewes on days 13, 16, and 20 after the induced oestrus in November using PGF2a (Lutalyse). In the light of the first results, the treatment was repeated on day 28 of the pregnancy in April and a second experiment using both PMSG+ and control sheep with either PGF2a or its

synthetic analogue (Cloprostenol) on day 20 was also carried out in February. In this way, the small number of animals were reused in the second experiment (chapter 2).

### **1.1.2 Animals studied**

Ewes: Scottish Greyface

A flock of 30 adult cast Scottish Greyface, which is a crossbreed of Border Leicester rams with Scottish Blackface ewes, were used for the present experiments. The sheep were bought from a dealer in October.

Rams

Five rams were used, one crossbred vasectomized and four entire rams of the breeds Blackface (#134), Border Leicester (#111), and Merino (Boroola gene heterozygote) and a Merino x Texel cross (LT).

### **1.1.3 Habitat and management of the animals**

Housing

The ewes were accommodated in a covered straw-bedded house for the duration of the study (1st November 1989 through 25th April 1990). The rams were kept separated from the ewes in another building of the same characteristics and only joined the ewes for mating or teasing.

Latitude

The experiment was conducted at the "Home Farm" of the University of Glasgow, which is located in Bearsden, Glasgow: latitude 55° 52'N, longitude 4° 14'W.

## Feeding

Hay was fed ad libitum (approx 3 bales per day per 30 ewes) together with a concentrate supplement (Ewe ball Cobs, BOMC) in the ratio of 1.5 to 2 lbs per ewe).

### 1.1.4 Synchronization of the oestrous cycle

Oestrus was induced by using intravaginal sponges of synthetic progestagens in cyclic sheep at three stages of the breeding season, namely November (n=30), Feb (n=29) and March (n=15).

In order to ascertain that all the flock (n=30) were cycling, a teaser ram, fitted with a harness and a crayon (SireSine), was introduced to the ewes on the 2nd of November before the first intended sponge insertion of the season.

Ewes were allocated randomly to the synchronization treatment both in Nov and February. Early pregnancies resulting from the 1st induced oestrus in November were interrupted by the administration of PG injections in the experiment that will be described in chapter 2, and therefore all the flock (n=29 at that time) was again available for the 2nd synchronization in February. The 3rd synchronization was carried out simultaneously with the study on abortion in early pregnant sheep (described in chapter II) and therefore only the non-pregnant animals (n=15) resulting from the 2nd synchronization were available this time for oestrous synchronization.

Synchronization and superovulation were combined together in the majority of the sheep. For this purpose a standard regime was used which involved administration of synthetic progestagens by sponges of 60 mg of medroxyprogesterone acetate (MPA, "Veramix", Upjohn Ltd) or 30 mg of Fluorogestone acetate (FGA, Cronolone, "Chronogest", Intervet Ltd) for 12 or 13

days and the superovulatory dose of 1500 I.U. Pregnant Mare Serum Gonadotrophin (PMSG, Intervet) as a single injection at sponge removal.

In the control sheep in February, the same progestagen treatment was utilized without using PMSG at sponge removal. However they were synchronized previously with a double prostaglandin injection 10 days apart as they were used in a control experiment for the synchronization with sponges which is explained in the next section.

#### **1.1.5 Matings**

The superovulated (PMSG+) ewes were mated at the first induced oestrus which was expected 24 hours after sponge removal and PMSG injection (Cameron, personal communication) or up to 48 hours (Gordon, 1983a). Rams were introduced 24 hours after sponge removal and left with the ewes for 2-3 days. In the control sheep, mating was designed to take place at the second induced oestrus after sponge removal. The ewe/ram ratio was always in the order of the recommended 10:1 or less (Gordon, 1983b).

#### **1.1.6 Detection of oestrus**

A vasectomized ram was used in the first half of the study (Nov-Dec and January) and a entire one (#134) at the end of it (Feb and March). The rams were fitted with ewe-marking crayons for cold weather (attached to a leather harness , Sire Sine), a method devised and described by Australian workers (Radford, Watson, and Wood, 1960). The colour of the crayon was changed every time that detection of oestrus was carried out. Lighter colours were used first and the darker ones were used at the end of the season, in the following sequence: yellow, red, blue, green and finally black. The marks

on the rump of the ewes were recorded either once (10 am) or twice a day (10 am and 5 pm) by running the ewes through a sheep race. The fitting of the harnesses was checked daily to ensure the comfort of the ram and that the crayon was intact and not covered by dirt. During oestrus detection the rams and ewes were accommodated indoors, in a space of approximately 6 x 15 m.

#### **1.1.7 Blood sampling**

Blood samples for plasma progesterone determination were collected from the jugular vein by venipuncture, using 19G needles into the heparinised collecting vessels (Monovette, Sarstedt). The samples were centrifuged within 30 minutes of the collection at 2000g for 20 minutes and the plasma separated from red blood cells. The plasma was drawn off into clean vials and stored frozen (-20°C) until assayed.

#### **1.1.8 Quantification of Progesterone: ELISA assay**

The plasma progesterone levels were quantified with a commercial kit (Ovucheck 96-well Plasma/Serum Progesterone EIA, Cambridge Life Sciences plc, Cambridge) based on the enzyme-linked immunosorbent assay (ELISA). The kit was first designed for the determination of progesterone in bovine plasma and later validated for most domestic species, including sheep (Eckersall & Harvey, 1987). Manufacturers claim an intra-assay precision at 1 ng/ml at 10% coefficient of variation and an inter-assay coefficient of variation of 25%.

Two pipettes ("Pipetman", Gilson Medical Electronics S.A.) of 10  $\mu$ l and 200  $\mu$ l were used to dispense the samples, the rest of the material and all the reagents were supplied in the kit. Plasma samples were assayed using the method recommended by the manufacturers. The

standard of 5 ng/ml was included between every 8 samples of the ewes' plasma and run as a control sample. An Elisa Plate/Strip reader (Titertek Multiskan Plus, Flow Laboratories ) set at 405 rpm, was used to read absorbance. The curve correlating the absorbance units of the standards (y) with their progesterone concentrations (x= 1.6, 3.18, 15.9 and 31.80 nmol/l) was then plotted using a computer (IBM PC) and the program Tittersoft E.I.A. Software version 2.0A (IBM). The progesterone concentration of the samples was also read by the computer from the standard curve. Finally, a printout was obtained of the graph followed by the values of the progesterone absorbances and concentrations.

## **1.2 CONTROL EXPERIMENT STUDYING SYNCHRONIZATION USING PROGESTAGEN SPONGES.**

### **1.2.1 Introduction**

Progesterone can be considered as the "organizer" of the oestrous cycle (Goodman, 1988; Haresign, McLeod, and Webster, 1983). If progesterone levels are high, tonic LH secretion is suppressed and consequently the preovulatory oestradiol rise that triggers an LH surge and in turn ovulation, cannot occur. If progesterone levels fall, tonic LH secretion increases and stimulates follicular development and the preovulatory oestradiol rise which would start the follicular phase events (Goodman, 1988).

Therefore the manipulation of the luteal phase allows a means of controlling oestrus. Progesterone administered exogenously during the breeding season can inhibit oestrus and ovulation which can be restored synchronously, 2-4 days after the cessation of the treatment (Dutt, and Casida 1948). Short-acting progestagens, ie FGA and MPA, capable of both

suppressing oestrus and inducing a synchronized oestrus have been developed (Robinson, 1979). These are administered intravaginally in impregnated sponges which allows fast progestagen withdrawal at will (Robinson, 1979).

Progestagens are left in the vagina for 12-14 days, which is the life span of a normal CL (Robinson, 1959). The purposes of this period is two fold: firstly, to allow enough time for the CL to regress so that the progestagen can take over its role, thus suppressing oestrus in all the possible stages of the cycle that may be present when sponges are inserted in a randomly cycling flock (Dutt, 1948; Hunter, 1980). Secondly, a period of 12 days of progesterone priming is believed to be necessary to induce a normal oestrus (Robinson, Moore and Binet, 1956).

Treatment with progestagens during days 6 to 13 does not change the CL function, as it does not reduce CL weight (Zimbelman, Pope, and Casida, 1959; Woody, Ginther and Pope, 1967b) nor progesterone output (Smith & Robinson, 1969) and corpora lutea regress at the expected time (Echternkamp, Bolt and Hawk, 1976). The administration of progestagens before the LH surge, prevented the development of the CL (Gaston-Parry, Heasman, Nemorin and Robinson, 1988). Progesterone when administered before ovulation produced a smaller CL (Woody et al., 1967b) and when administered on the day of oestrus, suppressed ovulation in some animals (Smith & Robinson, 1969) or in other animals, suppressed the production of progesterone (Smith & Robinson, 1969) and/or caused its premature regression (Smith & Robinson, 1969; Thwaites, 1971; Dixon & Twaites, 1973). During metoestrus, progesterone also undermined CL function, in term of weight, lifespan and progesterone output, although to a lesser extent than at oestrus (Zimbelman et al, 1959; Woody, First and Pope, 1967a; Woody et al., 1967b; Ginther, 1968).



The above reports on the suppressive effect of progesterone and progestagens on the formative phases of the CL would indicate that adequate synchronization of oestrus with progestagens can be expected when they are administered for the normal lifespan of the CL as proposed by Woody et al. (1967a). However there is also evidence to the contrary as progesterone treatment (Ottobre, Lewis, Thayne and Inskeep, 1980) or its analogue FGA (Gaston-Parry et al., 1988) did not inhibit the ability of the newly formed CL to secrete progesterone or shorten its lifespan (Gaston-Parry et al., 1988). Because of these contradictory conclusions, it was decided to carry out a small investigation to look at this area.

#### **1.2.2 Design, Material and Methods**

This auxiliary experiment was carried out to assess the effect of the insertion of sponges in cyclic ewes at different stages of their cycle, when the corpus luteum may be absent, developing or fully functional, to evaluate the possible interference during those phases of the CL development on the subsequent outcome of the synchronization. This was carried out to elucidate whether the observation of premature return to oestrus and low progesterone 6-8 days after sponge removal (Harvey, personal comm), was due to an outlasting of the spontaneous CL over the sponging period, or a short lifespan of the induced CL.

The experimental design consisted of the insertion of sponges on different known days of the cycle, namely; at luteolysis (d-1 and 0), at oestrous (d1), metoestrus (d2, 3, 4 and dioestrus (d5 and 6). Day 1 is designed to be day of oestrus to keep the consistency in all the chapters, as later day 0 will be considered the day of sponge removal. Peripheral plasma progesterone levels monitored the evolution of the corpora lutea following

the administration of the synthetic progestagen sponges.

The stage of the cycle at sponge insertion was determined by different means: either detection of the spontaneous oestrus by the use of teaser rams or by the induction of oestrus using a single or double injection of PG followed by oestrous detection by rams.

In a preliminary experiment (November), 14 ewes were used. In 7 of them (group 1), spontaneous oestrus was detected by rams and in the rest (group 2) oestrus was induced by a single injection of 125  $\mu$ g of the PGF<sub>2a</sub> analogue Cloprostenol (0.5 ml of Estrumate, Coopers Ltd.). Six pairs of ewes (one ewe from each group 1 or 2) were randomly allocated to the sponging on the first 6 days of the cycle, namely day of oestrus (d1), d2, d3, d4, d5 and d6. The two remaining sheep were used as non-sponged controls.

In January the experiment was repeated, this time synchronizing the cycles of 10 ewes with a double injection of prostaglandins (0.5 ml of Estrumate) ten days apart (Gordon, 1983b; Greyling & Van der Westhuysen, 1979). Blood samples were collected on the day of the 2nd injection and two days later to assess whether luteolysis had taken place. The first day when oestrus was detected by rams was designated as d1 and the sponges were inserted on the following days according to that: d-1 (48 h before the 1st oestrus detection ie 24 h after the 2nd PG injection), d0, d1 (day of oestrus), d2, d3 and d4.

### 1.2.3 Results

The progesterone levels in 4 of the ewes in which the detection of oestrus was the only means of determining the stage of the cycle, indicated that they were not on the days planned (d1, 2 and 3) when the sponges were inserted and therefore they were excluded from the results. Results from the remaining 22 ewes are presented in table 1.1.

Progestagens did not suppress neither the development nor the maintenance of the CL in the majority of the ewes (14/16) treated on days 1 (day of oestrus) to 6. The CL of these ewes secreted similar amounts of progesterone to the non-sponged controls (n=2), until day 8 which was the last day sampled.

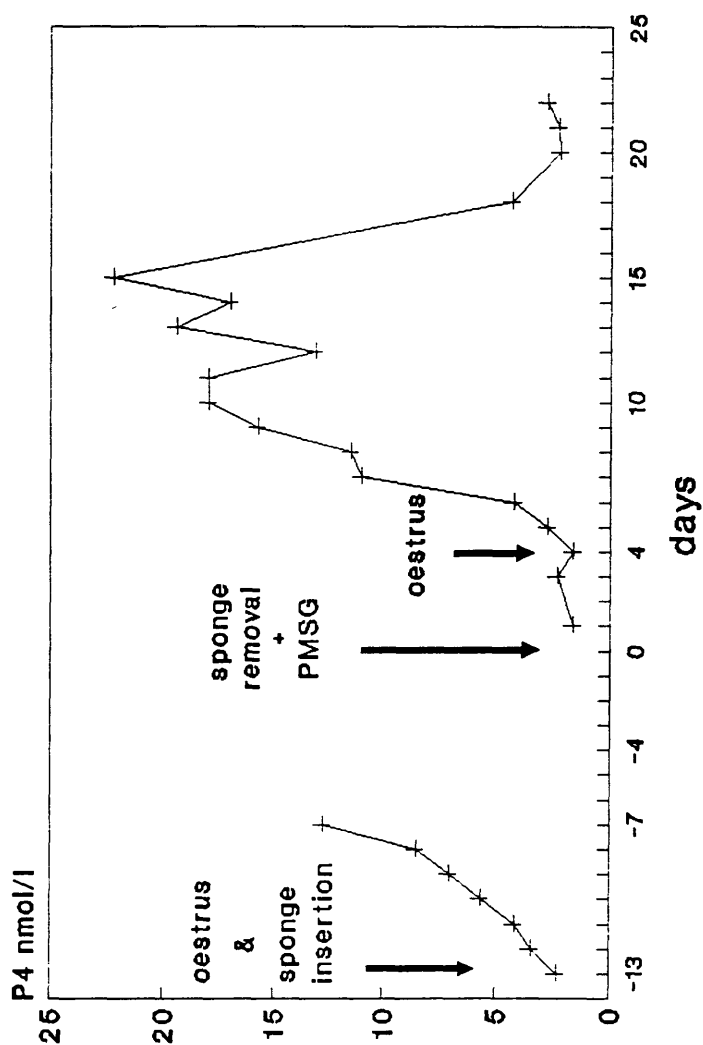
In two of the treated ewes (sponged on day 1 and 3) progestagen prevented the maintenance of the CL. Progesterone raised to luteal levels on day 4 and from there regressed to basal levels on days 5 and 6 respectively. The CL function after sponge removal of one of these ewes (#174) is shown in graph 1.1.

The ewes sponged before oestrus (days -1 and 0) did not show oestrus or CL development (n=4).

day of sponge insertion	ewes sponged n=	suppression of the CL n=	non- suppression of the CL n=	(**) time of sponging required	expected outcome with 12 days sponging
-1 (15)	2	2	0	<12	synchro- ny
0 (16)	2	2	0	<12	synchro- ny
1 (OE)	3	1	0	<12	asynchro- ny is possible
		0	2	14	
2	2	0	2	13	synchro- ny (**)
3	3	1	0	<12	synchro- ny
		0	2	12	synchro- ny (**)
4-6	8	0	8	12	synchro- ny
controls	2	0	2		
TOTAL	12	6	6		

Table 1.1: Effect of progestagen sponges, inserted on different days of the cycle on the CL function and its implications for oestrous synchronization. (\*\*) Assuming that the CL lasts only 12 days in a 16 day long cycle.

# Progestagen treatment for 13 days possible asynchrony in sheep #174



#### 1.2.4 Discussion

The objective of the present study was to clarify the importance of the different stages of the cycle at which progestagens sponges are inserted for synchronization of the cycle.

The main stages of the cycle in which the ewes could be found are:

- 1) Animals before oestrus ( d 15 = d -1 and d 16 = 0 ).
- 2) In the early luteal phase, during the CL formation (d 1 to 3)
- 3) During the advanced luteal phase ( d 4 to 13 ).

The basic principle of the synchronization of the cycle with progestagen sponges is that progestagens suppress oestrus and upon sponge removal and therefore rapid progestagen withdrawal, oestrus, ovulation and CL formation are stimulated at a fixed time (Robinson, 1979). This is achieved in two different ways, depending on the presence or absence of a CL during the progestagen treatment. If a CL is present, the progestagen treatment must outlast the lifespan of the CL in order to control the cycle (Hunter, 1980). Therefore regimes of 12 to 14 days of progestagen treatment are normally used, which corresponds with natural CL lifespan. When no CL is present, the duration of the treatment is not as critical as illustrated by the fact that 9 days of sponging is enough to synchronize anoestrous sheep (Cognie & Mauleon, 1983). However, anoestrus and the preovulatory period are obviously not totally equivalent.

None of the four sheep sponged with progestagens before oestrus, ie in the preovulatory period, exhibited oestrus and neither formed corpora lutea during the progestagen treatment. These results confirm the findings of previous work in which exogenous

progestagens administered in the preovulatory period abolished oestrus and the LH surge (Smith & Robinson, 1969; Gaston-Parry et al., 1988). Sheep sponged in that period will not ovulate, nor develop corpora lutea during the treatment and therefore a good synchrony could be expected with the usual regime of 12 to 14 days, or less, providing that enough days of progesterone priming are given to induce oestrus.

When the sheep were sponged on day 4 and onwards, i.e. during the mature stages of the CL, it developed normally in all of them (8/8). Because of the advanced stage of the CL, it is unlikely that the lifespan of these corpora lutea would outlive the routine treatment of 12 to 14 days with progestagens. Even in an hypothetical situation in which a ewe is sponged on day 4 for only 12 days, its CL will be on d 16 (4+12) at sponge removal and physiological luteolysis does not occur any later than that time in normal 16-19 days cycles (Zarco, Stabenfeldt, Quirke, Kindahl and Bradford, 1988).

Sponging early after oestrus (d 1 to 3) prevented the development of the CL in only 2/8 of the sheep in the present study. Smith & Robinson (1969) found that ewes sponged on the day of oestrus either did not ovulate or if they ovulated, the CL did not develop fully and regressed early. Using more modern technology to determine progesterone, Gaston-Parry et al. (1988) concluded that the CL develops normally if the progestagen is administered after the LH surge and fail to develop when this is given before the surge. In the present study, ewes sponged on day 1 (oestrus) may have been sponged either before or after the LH surge. The finding of CL suppression on day 3 in the present work, differs with the findings of Gaston-Parry et al. (1988) that administration of FGA after the LH surge has no significant effect on the CL function. This finding may indicate that the progestagen impaired the CL function

early after ovulation, as proposed by Smith & Robinson (1969). Alternatively, the synchronization of the cycle with PGs, before the insertion of the sponges may have induced premature regression of the CL on day 6 in this particular ewe.

The CL function in the 6 non-suppressed sheep, as judged by the peripheral plasma levels of progesterone, was maintained for at least 5-8 days. The levels of progesterone on those ewes were similar to the ones observed in the control ewes (n=2), suggesting that the CL became fully functional. These results indicate that the negative feedback of the progestagen on LH does not suppress the CL function in most cases which is in agreement with previous reports (Ottobre et al., 1980; Battista, Rexroad and Williams, 1984; Gaston-Parry et al., 1988). The maintenance of these corpora lutea during the progestagen treatment may compromise the outcome of the synchronization if they outlive the usual sponging period (12-14 days).

It has been well documented however, that the treatment with exogenous progesterone (Woody et al., 1967a; Woody et al., 1967b; Ottobre et al., 1980) or progestagens (Smith & Robinson, 1969) shortly after ovulation and before day 4 causes premature luteolysis, shortening the length of the cycle by 4 to 6 days. The shortening of the cycle after progesterone treatment is believed to be associated with the precipitous luteolysis of the CL (Ottobre et al., 1980) with earlier peaks of PGF<sub>2a</sub>. Sponging early after oestrus (d1 - d3) with a 12 to 14 day progestagen regime, would be successful if the CL that may develop during the treatment, regresses prematurely without outliving the progestagen treatment. In most of the sheep, the progesterone profile was only followed until day 6 or 8 in this study and therefore the occurrence of precipitous luteolysis that has been reported to occur on d 10-d12 (Ottobre et al., 1980) cannot be ruled out.



However in the studies of Gaston-Parry et al. (1988) the CL seem to have a normal lifespan after the progestagen treatment on the day of oestrus (day 1) or day 3. If this is the case, asynchrony may result with regimes of 12 -13 days of progestagens, as the developed CL may outlast the treatment.

The hypothesis that the development of a CL during the progestagen treatment will asynchronize oestrus when the ewe is sponged early in the cycle, was apparently confirmed in one case (ewe #174). The progesterone pattern during the MPA-treatment and after its removal, is shown in graph 1.1. This ewe was sponged on day 1 (day of oestrus) and developed a normal CL during the exogenous treatment of progesterone as shown by the luteal levels of progesterone in peripheral plasma. The luteal levels reflect the natural progesterone and not the synthetic progestagen (MPA) of the sponge, since the cross-reactivity between these two compounds is believed to be low in the assay (McPhee & Tiberghien, 1987) and low levels of progesterone were observed during MPA treatment in other ewes that experienced CL suppression. Therefore the progesterone is attributable to a functional CL during the progestagen treatment. The sponge was kept in the vagina of ewe #147 for 13 days. The onset of oestrus after sponge removal was out of synchrony with the rest of the sheep by about 3 days, occurring, as it did, 4 days after sponge removal and PMSG injection rather than in 1 day which is the expected time (Cameron & Harvey, personal comm). The ewe may have behaved as a non-sponged sheep, as indicated by various facts:

- 1) luteal levels of progesterone were raised after the sponge insertion as in non-sponged sheep, it is unknown whether the CL regressed prematurely or after the time of sponge removal.

2) The ewe was in oestrus 4 days after sponge removal, which is 17 days after the spontaneous oestrus at sponge insertion. This may indicate that she came into oestrus after spontaneous luteolysis (completed on d17) rather than after sponge removal. The oestrus behaviour was accompanied by very low progesterone levels (1.6 nmom/l), characteristic of oestrus levels (Eckersall and Harvey, 1987) and comparable to the levels shown by the speyed ewe in the present experiment using the same assay (1.7 nmol/l). This indicates that the ewe 4 days after sponge removal, was actually in oestrus rather than at metoestrus and therefore the ram's mark was not a false positive.

Because oestrus detection was not carried out on day 1, we cannot discard the possibility that she came into oestrus at the expected time, although this is unlikely. In that case, a well synchronized oestrus must have lasted for 4 days. Although oestruses lasting for up to 5 days have been reported, they are very uncommon and most sheep have oestrus for two days at the most (Cole & Miller, 1935; Gordon, 1983). Another possibility is that the onset of oestrus induced by PMSG and sponge removal, may have been delayed, as the intervals from PMSG-treatment to oestrus of three days or longer have been reported occasionally (Newcomb, 1976; Newcomb & Rowson, 1976; Evans & Robinson, 1980; Whyman & Moore, 1980).

3) The progesterone levels in the 2nd cycle did not reach levels higher than 23 nmol/l which is lower than the levels shown by pregnant sheep after the same regime of synchronization and superovulation (>31.8 nmol/l). The difference between these levels of progesterone may indicate that superovulation only occurred in the well synchronized group and not in the asynchronized ewe (#174). The pregnancy would indicate that those sheep were well synchronized with the sponges, were mated correctly and PMSG had stimulated

superovulation. Conversely, as a consequence of the existing CL in ewe #174, PMSG may have been injected too early to produce superovulation because the ewe did not come into oestrus at the planned time.

4) Finally, the progesterone of the 2nd cycle returned to basal levels 20 days after sponge removal, there were 16 days between the drop in progesterone and the detected oestrus 4 days after sponge removal. PMSG (Booth, Newcomb, Strange, and Rowson, and Sacher, 1975; Smith, 1988; Oyedipe, Pathiraja, Gyang, and Edqvist, 1989) or embryo mortality (Edey, 1979) may have prolonged the CL until day 20. The levels of progesterone however, were not particularly high in this ewe, and the lengthening of the cycle by PMSG is attributed to high levels of progesterone produced by multiple CL. A normal 16 day long cycle may have occurred following the oestrus observed 13 days before the sponge insertion, which suggests that the progestagen sponge failed to synchronize this ewe.

These observations warn that inaccurate synchronization may result from using progestagen sponges for only 12 or 13 days when the ewes are sponged early after oestrus, because a CL that lasts its normal lifespan may occur. In flocks that cycle randomly, at least 1/16 (6%) of the sheep can be expected to be found in oestrus and according to the present results, may result in non-synchrony with the rest. The effect could be even worse in a flock that has been exposed previously to the ram effect or any other means of synchronization. This possibility should be considered in any situation in which a precise synchrony is essential, as for AI programs or in the studies of particular stages of the cycle. Therefore sponging for 14 days is recommended rather than 12 days, and also the avoidance of the early days of the cycle, if known, for inserting the sponges.

### 1.3 STUDIES ON LUTEAL FUNCTION DURING DIFFERENT PARTS OF THE BREEDING SEASON.

#### 1.3.1 Introduction

The oestrus cycle of the female domestic mammals has been classically divided into 4 phases ("proestrus", "oestrus", "metoestrus", and "dioestrus"), using two criteria, namely the sexual behaviour of the female and the morphological changes in their reproductive tracts (Cupps, Anderson, and Cole, 1969). However in the ewe, the behaviour only allows differentiation of the two obvious phases seen in the presence of the male, that is, oestrus (with the acceptance of mating) and the inter-oestrus period (Lindsay, 1991).

At the ovarian level, the cycle can be divided into the luteal phase dominated by the presence of one or more corpora lutea and the follicular phase (2-3 days) (Lindsay, 1991) or interluteal period. The length of the cycle in the ewe depends mostly on the luteal phase, because is the longer phase of the cycle, lasting 13 days (Robinson, 1959) and also because the follicular phase is relatively constant in duration (Zarco et al., 1988; Bindon, Blanc, Pelletier, Terqui, and Thimonier, 1979; Cahill, Saumande, Ravault, Blanc, Thimonier, Mariana, and Mauléonet, 1981).

The average length of the oestrous cycle (or the interoestrus period) in the common breeds of sheep is 16.5 to 17.5 days (Quirke, Hanrahan and Gosling, 1979; Bindon et al., 1979; Robinson, 1959 quoting data from Asdell, 1946).

The duration of the cycle is very constant in that species (Cole & Miller, 1935; Goodman, 1988) with ranges of 14 to 19 days in the 90.4% of the sheep. This figure increases to 95% if cycles which are twice the normal length are also included (Goodman, 1988, quoting

data from McKinzie and Terril, 1937). The latter are considered to be two normal cycles in which the intervening oestrus failed to occur or was undetected. However other authors regard the interval of normal cycles to be 16-18 days (Cole & Miller, 1935) observed in 94 % (127/135) of the cycles and again considering 3 cases of "twofold" to have a normal cycle length.

Zarco et al. (1988) claims that most cycles are in the range between 15 and 18 days, with occasional exceptional cycles as long as 21 days. However MacKenzie & Edey (1975) found that cycles of more than 19 days were extremely uncommon, accounting for less than 1% of 500 cycles studied. Edey (1979) suggested that the limit of normality for the cycle length for embryo loss diagnosis purposes should be changed from 21 days to 19 days. It is important to determine this limit in order to differentiate embryonic loss from fertilization failure. Returns to oestrus earlier than 19 days postmating cannot separate conception failure from embryonic loss before day 13, but returns to oestrus later than 19 days would indicate that an embryo was present at days 12 to 13 thus preventing luteolysis and indicating that embryonic loss occurred later than this (Edey, 1967 and 1979).

Some small differences in cycle length of +1 day have been described among different breeds (Robinson, 1959), between age groups (Goodman, 1988) and also between strains and individuals (Zarco et al., 1988). Hafez (1952) quoted contradictory reports by other authors on breed differences and age differences. In his own study on British breeds during 3 successive breeding seasons, he found a higher incidence of multiple cycles within the mountain breeds and also age differences in cycle length. The individual variation was inconsistent and annual difference negligible (Hafez, 1952).

Great variability has been reported to be associated with the breeding season. In contrast with the regularity of the length of the oestrus cycle (16.5-17.5 days) described previously in the ewe, others found a disproportionately large number (32.5%) of abnormally long (>19 days) and short (<14 days) cycles in a 3 year study. These were apparently associated with both the beginning and the end of the season, suggesting that the cycle is only regular during the middle of the season and varies at the beginning and at the end (Williams, Garrigus, Norton and Nalbandov, 1956).

In physiological situations, progesterone appears to be secreted exclusively by the corpus luteum (Goodman, 1988 quoting Short et al., 1963 and Bjersing et al., 1972). Therefore progesterone secretion reflects the development, maintenance and regression of the CL. Concentrations rise from about day 4 of the cycle to a peak between day 7-8 and 13-14, then decrease rapidly to baseline on the day before oestrus (15-16) and ovulation (Robertson & Sarda, 1971; Hauger, Karsch and Foster, 1977).

However, clinically, only luteal (or high) and non-luteal (or basal) levels of progesterone can be easily differentiated by progesterone assays (Eckersall & Harvey, 1987). Both RIA and ELISA were developed to discriminate between progesterone and other steroids that may cross react with it (Sauer, Foulkes and O'Neill, 1982), specially oestradiol.

The plasma progesterone concentration is low or basal, (Thorburn, Basset and Smith, 1969; Robertson & Sarda, 1971), when no functional luteal tissue is present, namely during the follicular phase, on the day of oestrus and during anoestrus. When a CL develops, progesterone rises to luteal levels (>2.5 nmol/l) (Eckersall & Harvey, 1987) on day 3-4 of the cycle.

This distinction between luteal and non-luteal levels allows differentiation among a number of functional stages of the ovary: anoestrous from cyclic animals (Yuthasastrakosol, Palmer and Howland, 1975), follicular from luteal phase (Eckersall & Harvey, 1987) and pregnancy from non pregnant animals (Robertson & Sarda, 1971).

Variations to the general pattern described above have been reported such as the maximal concentration reached, which is depends on the breed (Quirke et al., 1979), the ovulation rate (Goodman, 1988), the time within the breeding season (Ward, 1986) and also individual variation in the general shape of the progesterone curve (Wilmot, Sales and Ashworth, 1985).

Higher levels of progesterone have been associated with heavier weights of the CL (Plotka, Erb and Harrington, 1970) and therefore with higher ovulation rates within the same breed (Emady, Hadley, Noakes, and Arthur, 1974; Wheeler & Land, 1977) or in different breeds (Bindon et al., 1979; Quirke et al., 1979). The clinical application of the maximal levels of progesterone was to attempt to correlate the number of CL with the maximum progesterone levels, but this has not been successful because the ratio is not totally linear, and a small increase in the secretion of progesterone follows a bigger increase in the number of CL (Quirke et al., 1979). Individual variations in the pattern of progesterone have also been described (Wilmot et al., 1985) with considerable variation in the interval from luteolysis to the next rise in progesterone.

Pregnant mare serum gonadotrophin (PMSG) at doses of 1000-1500 I.U. is widely used in superovulating sheep (Evans & Robinson, 1980; Smith, 1988) and under these regimes the levels of progesterone and oestradiol are augmented and their patterns grossly changed (Smith, 1988; Oyedipe et al., 1989; Booth et al., 1975).

However, EIA has been reported to be suitable to monitor the progesterone profiles under those circumstances. The EIA results at lower levels (<25 ng/ml), are also in considerable agreement with those of RIA in cattle (Boland, Foulkes, MacDonnell, and Sauer, 1985).

Positive correlation has been reported between the dose of PMSG and the number of ovulations when lower doses of PMSG (500-600 I.U..) were used. At doses greater than 800 I.U., the ovulation rate does not increase linearly and the percentage of ovulations per total follicular development decreases (Smith, 1988). The total progesterone concentration is raised in superovulated animals and is also positively correlated with the dose of PMSG (Oyedipe et al., 1989) and number of corpora lutea (Booth et al., 1975; Oyedipe et al., 1989). Other authors claim that it is best correlated with both the number of CL and the number of unruptured follicles, which may luteinize and produce progesterone (Evans & Robinson, 1980).

Progesterone increases faster after PMSG administration than in the normal cycle, luteal levels being reached between 1 to 3 days after oestrus, and these levels are equivalent or higher than the maximal in a normal cycle of the ewe and the cow (Oyedipe et al., 1989; Yadav, Walton and Leslie, 1988). The maximum is reached in the middle of the cycle (Booth et al., 1975) and the decline is delayed in comparison to the normal cycle, resulting in a longer cycle. The length of the cycle seems to be also correlated with the PMSG dose in sheep, the maximal length of the cycle being observed (24 days) with the highest PMSG doses (Oyedipe et al., 1989). Other effects of pathological character have been observed occasionally in the pattern of progesterone of superovulated animals. Oyedipe et al. (1989) reported the occurrence of double progesterone peaks in superovulated ewes, proposing that multiple



asynchronous ovulation may be causing them. A biphasic ovulatory response has been found in the goat (Cameron & Batt, 1991) and the cow (Calleson, Greve, and Hyttel, 1987). The LH activity of PMSG is believed to induce directly a premature ovulation before the sponge removal which is followed by the normal multiple ovulation after sponge removal (Cameron & Batt, 1991) or the absence of the LH peak and subsequent rise in progesterone (Calleson et al., 1987).

Other aberrations in the progesterone pattern include the premature regression of the corpus luteum which has been sporadically observed on days 3, 6 (Tervit, Allison, Smith, Harvey and Havik, 1976; Schiewe, Howard, Goodrowe, Stuart and Wildt, 1990; Schiewe, Fitz, Brown, Stuart and Wildt, 1991) and 12 (Robinson, Wallace and Aitken, 1989) in the ewe. This phenomenon has likewise been observed in the cow (Booth et al., 1975; Bouters, Moyaert, Coryn, Spincemaille, and Vandeplasse, 1980) and the goat (Armstrong, Pfizner, Porter, Warnes, Janson, and Seamark, 1982; Armstrong, Pfizner, Warnes, Ralph, and Seamark, 1983a & Armstrong, Pfizner, Warnes, and Seamark, 1983b; Stubbings, Bosu, Barker and King, 1986). It has been reported with both PMSG and other gonadotrophins (FSH-P and hMG) and is not apparently associated with the use of any particular one (Schiewe et al., 1990) in the sheep. In the goat PMSG, seem to be associated with a higher incidence than FSH (Armstrong et al., 1983b). It has been observed with both types of oestrous synchronization (progestagen sponges and prostaglandins). Although the incidence seems to be greater when the cycle is synchronized using prostaglandins in mid cycle (Tervit et al., 1976; Willadsen, 1979; Gordon, 1983a; Schiewe et al., 1991), it has been also observed when the cycle is synchronized with either MPA (Schiewe et al., 1991) or FGA (Robinson et al., 1989) progestagens. Variation of the incidence with the season have also been reported

in the ewe (Ryan, Maxwell and Hunton, 1987; Ryan, Hunton and Maxwell, 1991; Jabbour & Evans 1991; Jabbour, Ryan, Evans and Maxwell, 1991) and in the goat (Stubbings et al., 1986).

The Scottish Greyface is a cross between a hill bred female with a long anoestrous period (Scottish Blackface) and a breed of ram noted for prolificacy (Border Leicester). These types of crosses are very common in the upland farms of Scotland and North England and are bred after the Scottish Blackface ewe has produced about 5 crops on the hill. The upland farms are located at latitudes  $51^{\circ}$  to  $61^{\circ}$  N and height often over 200 metres above sea level (approx 600 ft) which limits the period of herbage growth, which commences in mid-April to early May and ceases by the beginning of October (HFRO, 1979).

Both parents, the Scottish Blackface and the Border Leicester, have short breeding seasons with a mean duration of  $139 \pm 9.4$  and  $131 \pm 11.7$  respectively (Hafez, 1952). This has been associated with their geographical origin at high latitude and altitude in Britain (Hafez, 1952). They are both deep anoestrous breeds and have a clear-cut annual breeding season. The Scottish Blackface has no activity in the ovaries both in terms of ovulation and even follicular growth during the summer (Ducker, 1974).

In the Scottish Greyface breed the mean duration of the natural breeding season reported is 149 days, starting their oestrus activity by the third week of September (22nd) and finishing in mid February (18th) (Ducker and Boyd, 1974). Therefore the centre of the breeding season in this breed (6th of December) precedes the shorter day of the year (22nd of December). However considerable variation between years in the onset of the breeding season has been reported in this breed with later dates (8th-24th of October) which displaces

the centre of the breeding season (9th-18th of December) closer to the shortest day of the year (Gunn, Doney, and Smith, 1979; HFR0, 1979). The ovarian activity is longer than behavioural activity of oestrus and most sheep experience one silent ovulation prior to the onset and after the cessation of the breeding season. In this transitory period between the breeding and the non breeding period and vice versa, at least 13% of the ewes have two or more silent ovulations. A definitive period of anovulation occurs in all the ewes around the longest day of the year (22nd June) but the exact duration has not been reported. Even the follicular activity seems to cease in mid-June and only very small follicles are found in June and July. This decrease in size is first noticed in April and increases again in August (Ducker and Boyd, 1974).

### **1.3.2. Material & Methods**

#### **1.3.2.1 Ewes**

Corpus luteum function after synchronization and superovulation was studied in a flock of 30 cycling cast Greyface ewes, at two different times within the breeding season namely at the peak during November-December and at the end in February - March.

#### **1.3.2.2 Synchronization and superovulation**

The cycles of the ewes were synchronized using intravaginal progestagen sponges, containing either 60 mg medroxyprogesterone acetate (MPA; Veramix, Upjohn Co) or 30 mg fluorogestone acetate (FGA; Chronogest, Intervet Labs Ltd, Cambridge, U.K.) inserted for 12 or 13 days in the majority. The sponges were inserted on known days of the cycle at the first synchronization and randomly in the last two inductions of oestrus during the second part of the study (Feb and March).

The stage of the cycle before the insertion of the sponges in the first period (Nov-Dec) was determined by oestrus detection with a teaser ram as is described later. On the day of sponge removal, which was called day 0 of the cycle, the animals were injected intramuscularly with 1500 I.U. of PMSG (Pregnant Mare Serum Gonadotrophin), (Intervet).

#### **1.3.2.3 Matings**

Fertile rams were introduced 24 hours after sponge removal and left with the ewes for 48 hour. The ewe/ram ratio was always less than 10 ewes per ram.

#### **1.3.2.4 Monitoring of the cycle**

The function of the CL was assessed by looking at two criteria: the length of the cycle (i.e. interoestrus interval) and the production of progesterone.

Teaser rams carried out oestrous detection from d4 to d16 in order to differentiate any cycle of shorter duration than 16 days. Oestrous detection was carried out as explained in 1.1.6. The marks were recorded once a day, supervising the mating behaviour of the ram and checking the crayons, to ensure that the ram was marking properly. The teasers used were vasectomized rams in the 1st part of the experiment (Nov-Jan) and entire rams in the 2nd part of it (Feb-April).

Blood samples for peripheral plasma progesterone levels determination were taken daily from day 1 to 16 of the cycle in the first period (Nov-Dec), but only on days 4, 5, 6, 8, 11, 14 and 16 in the second part of the experiment. Jugular venepunctures were carried out as described in 1.1.7.

The possibility that precipitous luteolysis was caused by prostaglandin release was assessed by the analyses of the plasma samples that were available around luteolysis for 13, 14-dihydro-15-keto-Prostaglandin  $F_{2-}$  ( $PGF_M$ ). Frequent blood samples (every two hours) were collected on days 5 and 6 in February and some of these were used to determine daily variation of  $PGFM$ .

Unfortunately, no enzyme inhibitor of PG synthesis and metabolism were added after sample collection because the time of the luteolysis was unpredictable and the sampling was not originally planned for these analyses. This preliminary run of PGs included:

(1) samples at luteolysis of the four sheep that definitely had short cycles (#162, #185, #119, #158).

(2) hourly samples from d6 of ewe #162 that had a short cycle of 10 days duration.

(3) two controls: a non-mated control sheep (#144) and a non-pregnant mated ewe with normal cycle length.

#### **1.3.2.5 Hormone Assays:**

##### **Progesterone**

Determined using the ELISA kit (Ovucheck Cambridge Life Sciences plc) as described in 1.1.8. In order to cut down costs and time, day 8 samples were tested first for progesterone in all the sheep, and only if any abnormalities were found in this preliminary run was the complete cycle of the particular ewe assayed.

##### **Prostaglandins**

13, 14-dihydro-15-keto-Prostaglandin  $F_{2-}$  ( $PGF_M$ ) was kindly analysed by Dr. Neil Harrison at the Medical Research Council (Edinburgh) using Radioimmunoassay.

### 1.3.3. RESULTS

The length of the oestrous cycle was considered to be the interval between the induced oestrus (next day after sponge removal and PMSG injection, (Cameron, personal communication) and the first day in which the next oestrus was detected by rams. Progesterone levels were assumed to reach basal levels one day before the next oestrus (Robertson & Sarda, 1971) for these calculations.

Cycles of non-mated sheep are normally classified into:

- 1) Normal being 14-19 days or its multiples.
- 2) Abnormal cycles are considered those single cycles that fall outside the normal range, being either short (<14 days long) or long (20-27) (Hafez, 1952).

In the present experiment, most of the animals were mated ewes (16/17 in Nov and 42/42 in the other periods) and therefore a slightly different classification was considered here, which could apply to both pregnant and non-pregnant sheep. Cycles were classified according to whether or not they lasted until the maternal recognition period (day 12) (Niswender & Nett, 1988). Cycles were therefore divided into short cycles (<12 days) or cycles longer than 12 days. The latter group comprised 4 types of animals: 1) pregnant females, 2) ewes whose cycles were of normal length in terms of normal returns to oestrus (14-19 days) and/or to basal levels of progesterone (day 13-18), 3) ewes studied only until day 13 and finally 4) long cycles with either delayed return to oestrus (>19 days) or to basal levels of progesterone (>18 days).

### **Length of the cycle (Tables 1.2 and 1.3)**

In most ewes (54/59) the cycle length was longer than 12 days. Despite the rather small number of cycles observed it was noticed that among the normal cycles (16-19 days), they tended to be longer (17, 18 & 19 days) at the end of the season (13/17) in February and March than at the peak in December (16 days) (2/5). There were more sheep with longer cycles (17-19) in February (10/11) than in March (4/6). Two long cycles were observed in Nov (#144) and in February (#119). The long cycle of November was observed in a non-mated ewe (#144). Oestrus was not observed within the expected 14-19 days and a peak of PGFM was noticed on day 16 with a subsequent slow drop in progesterone (Graph 1.2).

### **Short cycles: length, dates, progesterone patterns, incidence and stage of the breeding season.**

Premature progesterone drop was observed in 5 ewes after the induction of oestrus with MPA sponges and PMSG (5/43 or 11.63%) (Table 1.2). Three of them, observed at the peak of the season and in February, were definitively short cycles (<12 days) of 9, 11 and 12 days (Table 1.3). The other two cycles observed in March, were presumably of 5 days' length (#65 and #93). In the sheep that experienced short cycles, no significant peaks of 13, 14-dihydro-15-keto-prostaglandin F2a (PGFM) were detected, which remained at levels between 100-200 ng/ml (graph 1.3) on the days of the precipitous drop in progesterone. Similar levels of PGFM were observed in one of the ewes (#162) 3-4 days before the precipitous drop in progesterone. Short cycles occurred in all the stages studied within the superovulated groups, at the peak of the season (on the 5th and 6th of December) and in the transition period into anoestrus (one on the 24th of February and two on the 26th of March), at -17, -16, 65, and 94 days of the

Stage of breeding season	oestrus induced n=	type of proges- tagen (12-13d)	cycle following sponge removal	PMSG dose I.U.	short cycles n=
peak Nov-Dec 1989	6	FGA	1st	1500	0
peak Nov-Dec 1989	11	MPA	1st	1500	2
late Feb 1990	17	MPA	1st	1500	1
late Feb 1990	10	MPA	2nd	0	0
late March 1990	15	MPA 11 4**	1st	1500	2(**)
total of PMSG+ sheep	43	MPA	1st	1500	5
total	59				5

\*\* progestagens removed after only 9 days

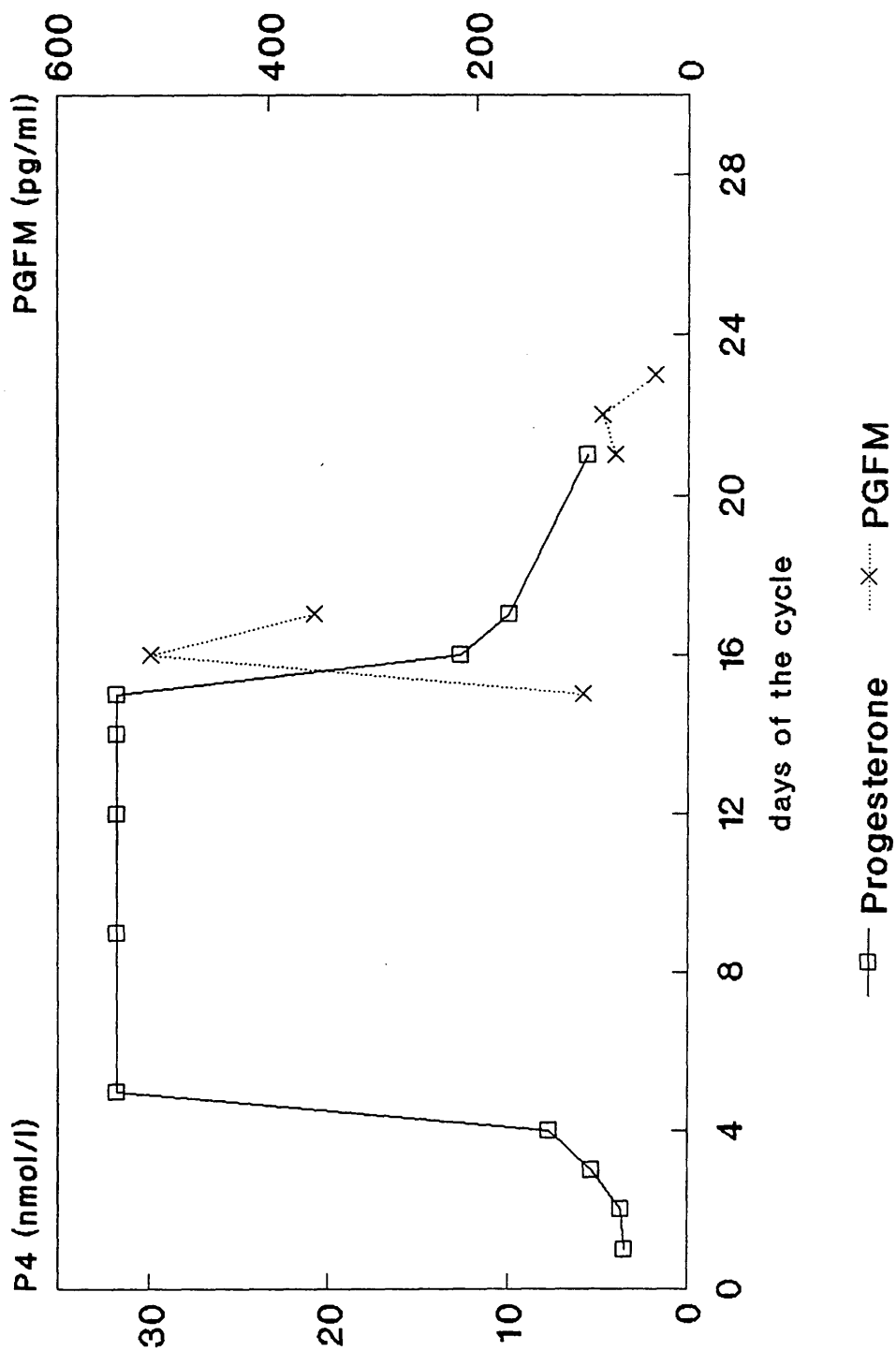
Table 1.2                      Frequency of short cycles (<12 days) following induction of oestrus using progestagens (with or without PMSG) related to stage of breeding season.



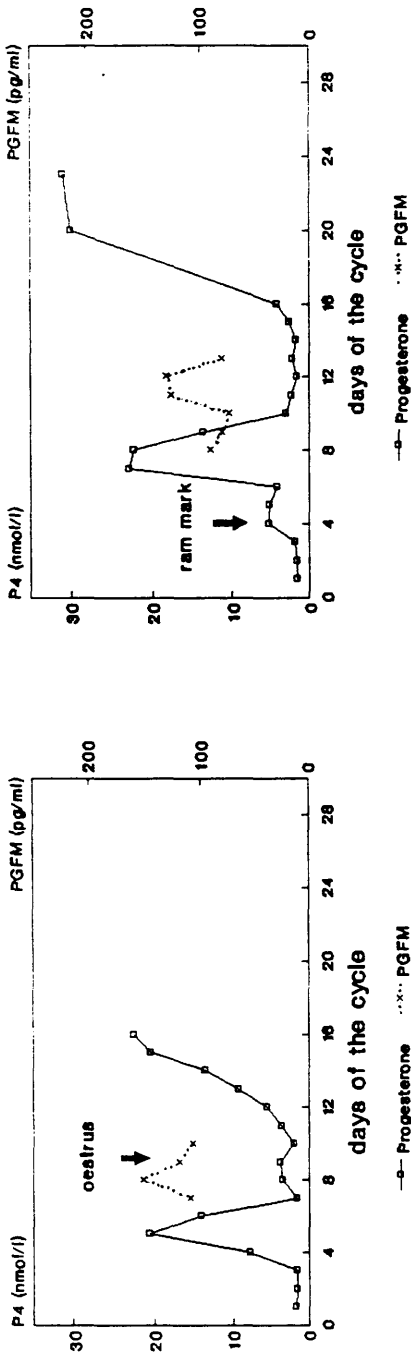
TIME OF YEAR	SHORT CYCLES (days)					NORMAL CYCLE LENGTH (days)					LONG CYCLES (days)		TOTAL n=
	5	9	11	12	16	17	18	19	>20	>23			
Nov-Dec (PMSG+)	-	1 #185	1 #119	-	2					1** #144			5
February (PMSG+)				1 #162		2	4	3	1				11
February (control)						1	1						2
March (PMSG+)	2 #93 #65					2		2					6

\*\* non-mated ewe

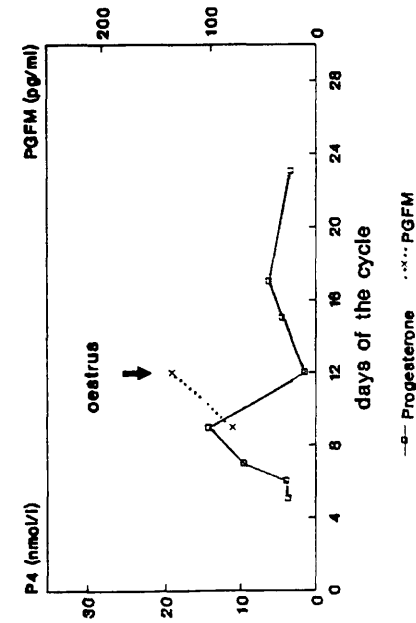
Table 1.3 Lengths of cycles observed in mated non-pregnant sheep



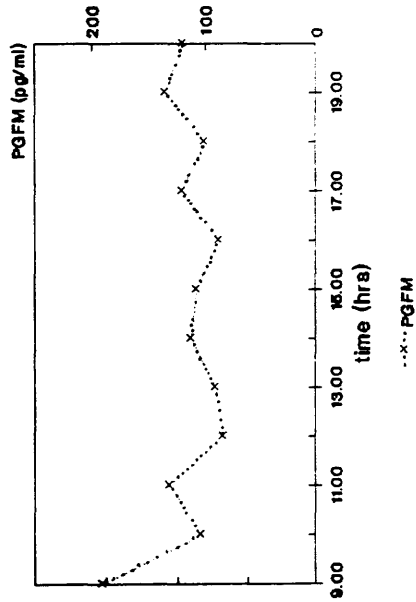
Graph 1.2 Long cycle (#144)



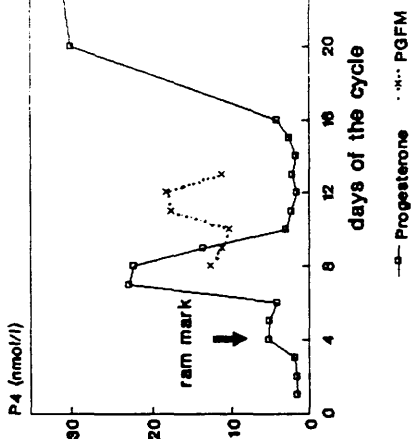
1.3a Ewe #185



1.3c Ewe #162



1.3d PGFM at day 6 (#162)



1.3b Ewe #119

Graph 1.3 Endocrine patterns in ewes showing short cycles

shortest day of the year (22/12) respectively. None of the ewes had more than one short cycle during the 3 periods of the study. Short cycles were absent in FGA-treated and superovulated sheep (n=6) and in non-superovulated ewes (n=10).

Endocrine patterns showing progesterone profiles (P4) and 13,14-dihydro-15-keto-prostaglandin F2a (PGFM) of each individual ewe (#185, #119 & #162) are shown in graph 1.3.

Ewe #185, which had a cycle of 9 days, presented a normal progesterone profile until day 5 (d0: sponge removal), when progesterone declined progressively from 21 nmol/l to basal levels by day 7 (2 days before oestrus).

Ewe #119 had a bimodal rise of progesterone, the first rise reached 5 nmol/l between day 4 to 6 and the 2nd, (23 nmol/l) on days 7 and 8 with a subsequent decline to basal levels on day 10. The ewe was marked on day 4 when she had slightly elevated luteal levels of progesterone. The colour of the crayon was not changed until day 16, and therefore if the ewe was marked again at the time of the premature drop in progesterone (on day 10), the mark could not be differentiated from the previous one on day 4.

Ewe #162 had a peak of 14.2 nmol/l on day 9 and from then progesterone levels declined to oestrous levels between days 10-12, oestrous was detected on day 12.

Ewes #65 and #93 (Not shown in graph 1.3)

Blood samples from these ewes were not collected as frequently and a drop from luteal levels on day 4 (8 and 5 nmol/l respectively) to basal levels on days 5 and 6, were suggestive of premature regression of the CL with cycles of 5 days long. Oestrus was detected only in ewe #93 on day 5. The ewes were sponged for

just 9 days in the belief that they were anoestrous, but the possibility of asynchrony due to a CL that may have outlasted the 9 days progestagen sponge cannot be completely ruled out.

#### 1.3.4 DISCUSSION

Early returns to oestrus towards the end of the breeding season (24,25 and 26th of January) were observed in a significant number of sheep (6/23 or 26%) after repeated superovulations (Cameron and Harvey, pers. comm.). This finding suggested that abnormal CL function involving a shorter lifespan may be common at the end of the season, however plasma samples between the oestruses or laparoscopy were not available to confirm it. Williams et al. (1956), using oestrus detection found that 32.5% of 2321 sheep experienced abnormally long (>19 days) or short (<14 days) cycles at the beginning and the end of the season. Similarly irregular activity of the ovary with short cycles (Lindsay, 1991) that resulted in more ovulations than oestruses (Land, Pelletier, Thimonier and Mauléon, 1983) was observed during both the end and the beginning of the next year's breeding season, but again individual ovarian activity was not described.

The CL function at the onset of the breeding season has been studied more extensively than at the onset of anoestrus. It is well documented that a brief increase in progesterone occurs just before the first normal oestrous cycle in the breeding season in most sheep (Thorburn et al., 1969; Yuthasastrakosol et al., 1975; Walton, McNeilly, McNeilly and Cunningham, 1977; I'Anson & Legan, 1988). The phenomenon has been frequently reported and studied at both the physiological onset of the breeding season, or in anoestrous sheep following artificial induction of ovulation by the "Ram Effect" (Oldham and Martin, 1979;

Knight, Tervit and Fairclough, 1981) or by GnRH (Legan, I'anson, Fitzgerald and Akaydin, 1985; McLeod, Haresign and Lamming, 1982; Hunter, 1991). Various mechanisms have been proposed to explain subnormal function, namely: inadequate preovulatory follicular development, decreased luteotropic support and a premature luteolytic stimulus (reviewed by Garverick & Smith, 1986). The current explanation for this phenomenon combines two findings, first that progesterone priming (McLeod et al., 1982; Southee, Hunter and Haresign, 1988a) and second hysterectomy, (Southee, Hunter, Law and Haresign, 1988b) both prevent premature CL regression (Hunter, Ayad, Gilbert, Southee and Wathes, 1989). When ovulation is induced in anoestrous animals, the absence of prior progesterone priming, may allow luteolysis to be stimulated by waves of the developing follicles during the induced luteal phase. This would have no effect in the ewes primed by progesterone, which possibly acts by two mechanisms: 1) by enhancing the effect of subsequent progesterone on the inhibition of oxytocin receptors and 2) reducing the secretion of PGF2a in response to the stimulus of oxytocin and/or oestradiol (reviewed by Hunter, 1991).

The study of the CL function at the end of the season is interesting in order to clarify the mechanisms responsible for its inadequacy. Progesterone priming obviously exists from the numerous previous cycles, thus making the above explanation unsuitable for the phenomenon at the end of the season. In addition, as was expressed before, there is a lack of information on the CL function of individual sheep at the end of the season and the onset of anoestrus itself.

The present work was undertaken to study the occurrence of abnormal CL function at the peak and at the end of the season, to see if the above observations were a consistent finding, to evaluate the seasonal effect and other possible factors involved that may open further

investigations on that topic in the future. The work was obviously limited by the unpredictability of this phenomenon within the individual ewes in the flock.

The induction of oestrus with MPA and with a superovulatory dose of PMSG in cyclic Scottish Greyface ewes, increased the incidence of short cycles in the present work. The total incidence after the induction of oestrus regardless of the stage in the breeding season was 11.63% (5/43), which is similar to Cameron's findings in 1987 (pers. comm.) using the same regime of oestrus induction with Scottish Greyface ewes, (11.86% or 7/59). It is not clear whether the drop in progesterone observed five days after sponge removal in two ewes in March (#65 and #93), was due to asynchrony or premature regression. If these two ewes were still cyclic at mid March and developed a CL during the 9 day sponge treatment, the drop in progesterone observed may correspond with its luteolysis. However, if they were anoestrus, the drop in progesterone indicates subnormal function of the induced CL. Excluding the ewes sponged for only 9 days (n=4), 3 out of 39 (7.7%) presented abnormal CL function after the induction of oestrus with MPA and PMSG in the present work. These figures (7.7%-11.63% and 11.86%) are higher than those reported for the spontaneously cycling adult sheep of various British breeds including the Scottish Blackface and Border Leicester (0-3% of range with an average of 1%) (Hafez, 1952). The consistency and the higher incidence of short cycles observed when the cycles are induced by MPA and PMSG as opposed to the natural ones, may indicate that the induction of oestrus by PMSG in the circumstances of the present experiment increased the incidence of short cycles. The different circumstances of the synchronization that may have contributed are namely: 1) type of synchronization, 2) progesterone priming 3) PMSG itself and 4) stages of the breeding season.

Schiewe et al. (1990) has proposed that the type of gonadotrophins (PMSG, P-FSH and hGM) used does not affect the incidence of short CL in sheep, but the type of synchronization employed does. He and other authors (Schiewe et al., 1990; Schiewe et al., 1991; Tervit et al., 1976; Willadsen, 1979 and Gordon, 1983a) had reported very high incidences (30-60%, 41%, 50% or more and 87.5%) of short CL when PG were used at mid cycle for oestrus synchronization in superovulatory regimes. The direct comparison of either a double injection PGF-2a or MPA for oestrus synchronization with P-FSH, showed that a higher incidence of short cycles was related to the use of PGF2a (7/8 vs 1/8) (Schiewe et al., 1991). These authors, using a superovulatory regime which consisted of a combination of MPA and P-FSH but with a different breed and latitude, found a slightly higher but similar figure (12.5%, 1/8) to the present findings (7.7% to 11.63%). Jabbour & Evans (1991) also found a similar figure (10%) using MPA and 1200 I.U. of PMSG. Robinson et al. (1989) found a smaller incidence (5.4%), using FGA in combination with different regimes of gonadotrophins (PMSG + GnRH and P-FSH) with both Scottish Greyface and Blackface breeds. In the present work, FGA was used in 6 animals and none of them presented short cycles. However, the number is too small to withdraw conclusions about the differences in the incidence of short cycles when one or the other progestagen is used. A direct comparison may be interesting, as it is possible that the use of FGA may be associated with a lower incidence of short cycles in Robinson et al.'s and the present findings.

As was pointed out previously, the use of progestagens before the induction of oestrus in anoestrous sheep, with either the "ram effect" or with multiple injections of GnRH, restores the normal function of the CL (Oldham, Cognie, Poindron and Gayerie, 1980; McLeod et al., 1982; Pearce, Oldham, Haresign and Gray, 1987; Hunter, Southee and Haresign, 1987).



On the other hand, CL function was found to be apparently normal in deep anoestrous ewes (Robinson et al., 1989) and goats (Stubbings et al., 1986) when synchronized with progestagens in superovulation programmes. In contrast, in the same studies, the identical superovulatory regime was associated with premature regression of the CL. It is intriguing that the luteal function may be better when oestrus and superovulation are induced in anoestrous sheep than during the breeding season.

If progesterone priming determines the time of luteolysis in the subsequent CL (Goodman, 1988), the above observations may indicate that, when a superovulatory regime is initiated, the presence of corpora lutea in cyclic females may interfere with the function of the induced CL. Certain stages of the CL may be more problematic, explaining why only a number of animals exhibit short CL. If progesterone priming is essential for a normal CL function, a minimum of days is required (Hunter et al., 1987) but also overlong priming may be detrimental for the CL function. A overlong period of progesterone priming may be produced when ewes are sponged late in the luteal phase, as the endogenous production would be followed by the exogenous progestagen. Lower fertility has been observed with prolonged treatments of progesterone in ewes (Wishart, 1967; Laster & Glimp, 1974) and cattle (reviewed by Odde, 1990). In the present work, a control cycle is not available for all the ewes, but the two ewes that had a short CL in November had their sponges inserted on days 3 and 10 of their oestrous cycles. Only the one sponged on day 10 would fit with the above hypothesis. In the ewe sponged on day 3, endogenous progesterone and exogenous progestagen lasted for the same period. The endogenous production of progesterone, compared with the potency of the synthetic progestagen suggests that an increase in the

production of progesterone during the sponging period by the CL will probably make no difference.

Short cycles were only observed in the superovulated groups and were absent among the controls (n= 10). The absence of short cycles in those sheep may not be significant, considering the small number of ewes that were studied (n= 10) and the fact that the incidence of short cycles was also smaller in the superovulated sheep during the same period of the breeding season (1/17). Therefore there is not enough evidence in the present experiment to confirm the association between short cycles and the use of PMSG. However, an incidence of 5-20% of short cycles seems to be associated with the use of progestagen sponges and PMSG to induce superovulation (Robinson et al., 1989; Cameron, pers comm; Jabbour & Evans, 1991; Schiewe et al., 1991). Various mechanisms have been proposed to explain how PMSG may produce CL insufficiency. PMSG administration hastens the LH surge (Evans & Robinson, 1980) which in turn may result in an over-short follicular phase (Garverick & Smith, 1986) and the number of granulosa cells and LH receptors of the preovulatory follicle predetermine the subsequent steroidogenesis of the CL (Niswender & Nett, 1988; Hsueh, Adashi, Jones and Welsh, 1984). On the other hand there is evidence of the involvement of PGs in the early luteal regression of superovulated goats, as the administration of PG inhibitors prevent the premature demise of the CL (Battye, Fairclough, Cameron and Trounson, 1988). Uterine involvement has also been suspected in the ewe (Willadsen, 1979). PMSG may induce the secretion of endogenous prostaglandins arising from the uterus by two mechanisms namely: by stimulation of follicular growth and steroidogenesis during the early luteal phase (Armstrong et al., 1982; Booth et al., 1975) or by inducing premature ovulations (Evans & Robinson, 1980) which would result in high levels of progesterone on the first 3 days after oestrus (Battye et al.,

1988). Both the administration of progesterone in the first 3 days after oestrus in sheep (Ottobre et al., 1980) or oestradiol in the early luteal phase in cattle (Wiltbank, 1966) or during mid-luteal phase in both sheep and cattle (Hixon, 1987) can shorten the cycle.

Plasma progesterone samples on days 1 to 4 were only available on 2 of the 5 ewes that experienced short cycles in the present study. These two ewes (#185 and #119) did not have luteal levels of progesterone before day 4, which indicates that progesterone did not induce earlier luteolysis in those animals. Furthermore, other ewes in the present study had luteal levels of progesterone in the first 3 days after sponge removal and had normal or long cycles (#144 and #158).

The lifespan of the short cycles observed in the present experiment was slightly longer than some previous reports (9-12 days vs 4-6 days) (Schiewe et al., 1991; Jabbour & Evans, 1991). Oestrogens stimulate PGF<sub>2a</sub> secretion if given late (days 9-19), but not if given early (days 4-5), during the oestrous cycle (Goodman, 1988). This indicates that luteolysis caused by oestrogens may have been possible in the days that were observed in the present work, especially on those with longer cycles, as progesterone takes 48 h to decline after oestrogen administration (Hixon & Flint, 1987).

Only in ewe #144 was a clear peak of PGFM detected (>512 pg/ml) at luteolysis. The levels of PGFM found around the progesterone drop in the rest (n= 5), both those with short cycles and normal ones, were not higher than 200 pg/ml. This is higher than basal levels (50-60 pg/ml) reported by other authors (Hixon & Flint, 1987; Zarco et al., 1988). Peaks of similar magnitude or higher, have been reported on days 9-10 after oestrogen-induced luteolysis (Hixon & Flint, 1987) and on days 12 to 16 at physiological luteolysis (Zarco et

al., 1988; Flint & Sheldrick, 1983). Luteolytic peaks of PGFM seem to vary in height considerably at normal luteolysis (Hixon & Flint, 1987; Zarco et al., 1988). The mean concentrations of PGFM increased from approximately 60 to 80 pg/ml in abnormal CL induced with GnRH in anoestrous sheep (Hunter et al., 1989).

According to the above findings, the moderate levels of PGFM (100-150 pg/ml) observed in the ewes which experienced short cycles in the present study, may indicate that precipitous secretion of PGF-2a and luteolysis took place. However in the present circumstances of the collection of the samples, these figures cannot be claimed as peaks of PGFM. The levels observed 4-6 days before the drop in progesterone on ewe #162 were also between 84 to 136 pg/ml. These levels may or may not be basal as PGFM is known to rise and start peaking 4 days before luteolysis in the normally cycling ewe (Flint & Sheldrick, 1983; Zarco et al., 1988). Furthermore, in some of the goats (2/4) that experience premature luteolysis on day 7, PGFM has been observed to be high as early as 2-3 days after oestrus (Battye, Fairclough, Cameron, and Trounson, 1988). In conclusion, the levels of PGFM observed in the present experiment in association with short cycles are compatible with the occurrence of premature luteolysis, although they are not full evidence of it.

In the present work, 2 out of the 3 or 5 short cycles observed occurred on the 5th and 6th of December, at the peak of the season (Ducker and Boyd, 1974) which is believed to precede the shorter day of the year (around the 22nd of December) in this breed. A striking observation was the consistency between the dates in which the CL regressed prematurely in two years, 4th of December in 1987 (Cameron, pers comm) and on the 5th and 6th in the present work in 1989. The percentage of ewes regressing prematurely at the peak of the season was also similar in both years (11.11% or 1/9 vs

11.76% or 2/17 respectively). Seasonal variations have been reported in both the production of oestrogen and the sensitivity of ewes to it, which was maximal near the middle of the breeding season (Fletcher & Lindsay, 1971). The ovarian response to PMSG is also known to be higher at the peak of the season. Follicles in PMSG-treated ewes at the peak of the season have been reported to produce oestrogens more intensively than in anoestrus (Evans & Robinson, 1980). In keeping with one of the above hypothesis, hyperstimulation of follicles by PMSG which in turn produces excessive amount of oestrogens may have brought about premature luteolysis in the present study (Armstrong et al., 1982). It is possible that a higher incidence of short cycles at the peak of the season may be explained by the augmented steroidogenic action of PMSG at this time of the season.

A similar seasonal effect was observed by Ryan et al. (1987) in Australia, using different doses of PMSG and/or FSH-P, the incidence of prematurely regressing CL was higher at the peak of the season in Autumn (May) than in Spring (Nov) (24% vs 6%). However this group associates the seasonal effect with changes in the nutritional status (Ryan et al., 1987; Jabbour & Evans, 1991). Supplementary feeding with lupins reduced the incidence of premature luteal regression despite increasing oestrogens levels (Jabbour et al., 1991).

The present findings are also in line with the findings of Stubbings et al. (1986) in various breeds of dairy goats (Alpine, Saanen and a cross from both breeds). Luteal failure occurred more frequently during the breeding season than in the transition period to anoestrus (2/4 vs 2/9) and was absent during the seasonally anoestrus period (0/5). The higher incidence of short cycles in the middle (French Alpine breed) or end of the season (Creole breed), after induction of oestrus with FGA and PMSG for AI, have been associated

with the breed and the genetic characteristics of the breeding season (Corteel, 1973; Corteel, 1977). The Alpine goat is a deeply anoestrous breed with a breeding season similar to the Scottish Greyface ewe, whereas the Creole is a subtropical breed and 2/3 breed during all the year.

Most authors report the occurrence of short cycles in periods of transition between acyclic and cyclic period and vice versa; at the beginning and end of the breeding season, after postpartum anoestrus and at the onset of puberty (Lauderdale, 1986). The cause is unknown, but hormonal imbalance resulting in lack of luteotrophic support of the newly formed CL has been suggested (Garverick & Smith, 1986). The seasonal effect on the CL function could be related to deficiencies in gonadotrophins in the transition periods of the season because the LH pulse frequency is known to be reduced during anoestrus (Karsch, 1984) and this hormone is believed to be the essential gonadotrophin in the ewe (Niswender & Nett, 1988). In the present work however, a number of findings do not support the hypothesis of a seasonal involvement with lack of gonadotrophins at the transitional periods of the breeding season.

1) The incidence of short cycles after the induction of oestrus with MPA and PMSG was higher at the peak of the season, (in Nov-December) than at the end of it (in February and March) (2/11 vs 1/28-3/32). In contrast, Cameron & Harvey (personal communication) found a much higher incidence of short cycles in the Scottish Greyface towards the end of the season in January than at its peak in December (1/9 vs 6/22), which suggested a possible exhaustion of gonadotrophins at the end of the season. However, these results were not reproduced in the present experiment. Robinson et al. (1989) also found no difference between the incidence during the breeding season in January or at the end of it in

February (5% vs 5.8%) on the Scottish Greyface and Blackface in a similar latitude

2) Short cycles were consistently found at the peak of the season (4, 5, 6th of December) and in the same percentage in two years; 11.11% (1/9) in Cameron's work (1987) and 11.76% (2/17) in the present experiment.

3) Absence of short cycles at the beginning of the season. Cameron studied the returns to oestrus early in the breeding season (28th October to 28th November) and found that all the cycles were of normal length (15-19 days) in the 19 ewes observed in that period, using the method of oestrus induction used in the present experiment.

4) The maximal incidence was observed in January before the end of the season (26%) by Cameron, whereas in the present experiment a lower frequency (10%) was found later in the breeding season (February-March). This may indicate that the incidence of short cycles does not increase with the advancement of the breeding season in the transition to anoestrus or that both years are not comparable.

The present findings that 2 out of 5 short cycles observed during the periods studied, occurred at the peak of the season (in early December), and taken together with Cameron's findings that short cycles were more frequent at the peak of the season than earlier (Oct-Nov), would suggest that lack of luteotropic support happened in the peak rather than earlier in the season. This does not support the theory that the incidence of short cycles is associated with seasonal deficiencies of gonadotrophins, as LH pulse frequency increases from anoestrus to the peak of the breeding season (Goodman, 1988; Karsch, 1984).

At the end of the season there is evidence for both the acceptance and the rejection of the hypothesis that seasonal effects on LH are associated with the incidence of short-lived corpora lutea. On the one hand, the present experiment did not reproduce the pattern found by Cameron, when the peak of the season is compared with later. However on the other hand, a higher proportion of short cycles may have been present in March (2/15) than in February (1/17) in the present work, which would be in line with Cameron's findings. It must be said that the occurrence of the two short cycles in March should not be over-emphasized because the evidence that they were indeed short, was not absolute, as has been already explained. All the ewes were cyclic in February and some of them even in March, indicating that the breeding season was prolonged with the PMSG treatment. This may explain the low incidence of short cycles observed in February in the present experiment, because the lack of gonadotrophins could probably be expected later.



## Chapter 2

### STUDIES ON TERMINATION OF EARLY PREGNANCY USING PROSTAGLANDINS

#### 2.1 INTRODUCTION

Progesterone is the essential gonadal hormone required for the maintenance of pregnancy after the first two days in the ewe. This has been shown by experiments involving its administration to ovariectomized (Foote, Gooch, Pope and Casida, 1957; Moore & Rowson, 1959; Bindon, 1971) or to both ovariectomized and adrenalectomized ewes (Cumming, Baxter, and Lawson, 1974). During the period from conception to the first 55 days, the production of progesterone and maintenance of the pregnancy depends solely on the corpus luteum (Casida & Warwick, 1945; Denamur & Martinet, 1955). From day 55 and onwards the production of progesterone by the placenta (Rickets and Flint, 1980) is sufficient to support pregnancy and therefore castration after then does not result in abortion (Denamur & Martinet, 1955 ; Casida & Warwick, 1945).

The use of the luteolytic drug Prostaglandin F-2a (PGF-2a) or its analogue Cloprostenol (Baird & Scaramuzzi, 1975) have displaced other methods of early pregnancy termination in most domestic species (Gordon, 1983c,d,e, and f; Barth, 1986; Ott, 1986; Lofstedt, 1986), because of their safety, efficacy and easy of administration.

In the ewe, the induction of luteolysis can terminate pregnancy solely during the period in which the CL is the only source of progesterone, ie before day 55. Although the sensitivity of the CL to the luteolytic action of PGF-2a varies throughout this period of pregnancy. The injection of 6-8 mg prostaglandin F2a

(PGF2a) or 125-250  $\mu$ g of its synthetic analogue, Cloprostenol cause precipitous luteolysis in cyclic ewes (Arthur et al., 1989). The cyclic CL is responsive only between days 5 and 14 after oestrus (Acritopoulou, and Haresign, 1980). However the sensitivity to luteolytic drugs appears to be altered in early pregnancy. The administration of PGF2a by different routes is less effective in inducing luteolysis in pregnant than in nonpregnant ewes on days 12 and 13 (Inskeep, Smutny, Butcher and Pexton, 1975; Mapletoft, Lapin and Ginther, 1976; Pratt, Butcher and Inskeep, 1977; Silvia & Niswender, 1984). Likewise, the administration of Oestradiol-17 $\beta$  in luteolytic doses for cyclic ewes is only partially effective in early pregnant sheep, on days 11, 12 and 13 (Kittok & Britt, 1977). Silvia & Niswender (1984) established the minimum dose of an IM injection of PGF2a capable of producing luteolysis on day 13, this being smaller for nonpregnant ewes (4 mg) than in pregnant ewes (6-10 mg) with one or two CL respectively. Nancarrow Evison and Connell (1982) also found higher resistance to Cloprostenol in ewes that had multiple corpora lutea and because this effect of the number of CL was only seen within the pregnant ewes and not in the cyclic ones, he concluded that the number of CL reflected the number of conceptuses and therefore the resistance was attributable to a higher embryonic mass.

The resistance of the pregnancy CL to luteolysis has been described by Silvia and Niswender (1986) as a transitory phenomenon, resistance being absent on day 10 after mating, developing and being greatest between days 13 to 16 and again absent in most ewes by day 19. This transient pattern of resistance to PGF2a coincide with the pre-attachment period (Guillomot, Flechon, and Winterberger-Torres, 1981; King, Atkinson and Robertson, 1982). During this period the trophectoderm of the blastocyst undergoes a dramatic elongation

(Rowson and Moor, 1966) and represents the first interface between the conceptus and the uterus (Ashworth & Bazer, 1989). The embryo has to signal its presence in the uterus to rescue the CL from luteolysis and establish pregnancy. This is known as "maternal recognition" of pregnancy and occurs on days 12-13 in the ewe (Niswender & Nett, 1988). Therefore the resistance to exogenous luteolytic drugs during early pregnancy is thought to be mediated by similar mechanisms to those responsible for luteolysis annulation in the maternal recognition of pregnancy. The capacity of the embryo to prevent luteolysis has been related by McCracken Schramm and Okulicz (1984) to either (a) suppression or alteration of PGF2a release by the uterus (antiprostaglandin secreting effect or antiluteolytic effect) or (b) the direct protection of the CL from the luteolytic effect of PGF2a (luteoprotective effect). From day 12 to day 16 the conceptus secretes substances such as an antiluteolytic protein (ovine trophoblast protein-1 or oTP-1) (Martal, Lacroix, Loudes, Saunier and Wintenberger-Torrès, 1979; Godkin, Bazer, Moffatt, Sessions, and Roberts, 1982) and the luteoprotective agents PGE-2 and PGI-2 (Henderson, Scaramuzzi and Baird, 1977; Marcus, 1981; Silvia, Fitz, Mayan and Niswender, 1984a; Silvia, Ottobre and Inskeep, 1984b; Vincent & Inskeep, 1984).

There are number of reports on resistance of the pregnancy CL to exogenous luteolysins during the postattachment period (on days 20, 23 and 33), in contradiction with the absence of resistance found by Silvia & Niswender (1986) beyond day 19. He proposed that this difference between his work and Nancarrow et al.'s (1982) finding of resistance on day 21, may have been due to the absence of superovulation in his own experiment. However although in smaller percentages, resistance has also been found in non superovulated ewes in this period by others (Reid & Crothers, 1980;

Tyrrell, Lane, Nancarrow and Connell, 1981). Estrumate has been reported to be unsuccessful in producing luteolysis in variable percentages, ranging from 4% to 30.7% on days 20-23 (Reith & Crothers, 1980), 33% between days 11-29 (Bottomley, personal communication in Reith & Crothers, 1980) and 8% between days 20 and 60 (Tyrrel, 1981). A higher percentage of resistance (65%) was found in day 21 when different doses of PMSG (400, 700 and 1000 I.U.) were used to produce a range of ovulation rates (Nancarrow et al., 1982). The resistant ewes to the abortive treatment corresponded with the ones that had higher ovulation rates. Resistance to PGF<sub>2a</sub> and to Oestradiol-17 $\beta$  on day 20 (Lacroix & Kann, 1986) has also been reported; all the ewes were resistant to Oestradiol-17 $\beta$  luteolytic effects and a very high (80%) proportion of ewes presented resistance to this drug as late as on day 33.

## **2.2 Material and Methods**

### **2.2.1 Experiment 1: Lutalyse administered at different times of the pregnancy (Table 2.1).**

A total of 24 ewes were synchronized in November and March, using a 12 day regime of FGA sponges (d0 = day of sponge removal), superovulated with PMSG (1500 I.U.) and mated as described in General Material and Methods (chapter 1). Pregnancy diagnosis was carried out by measuring plasma progesterone levels on day 16 (Robertson & Sarda, 1971) and the pregnant ewes (n= 18) were then randomly allocated to the luteolytic treatment of an intramuscular injection of 2, 3 or 4 ml of Lutalyse (10, 15 or 20 mg respectively of Dinoprost tromethamine, Upjohn Ltd.) on either day 16, 20 or 28 of pregnancy (see table 2.1, page 53). A group of 6 ewes (group 1) were treated on day 13, but at this stage as it was not possible to diagnose pregnancy because luteolysis had not started, they were treated with Lutalyse without knowing their pregnancy status.

### **2.2.2 Experiment 2: comparison between Lutalyse and Estrumate (Table 2.2: groups 1 to 4)**

Oestrus was induced in February with (n=17) or without (n = 10) PMSG, then the ewes were natural mated as described in General Materials & Methods (Chapter 1). Pregnancy diagnosis was also carried out by measuring plasma progesterone levels on day 16. Sheep on day 20 of pregnancy were chosen after the 1st oestrus induction and superovulation (PMSG +) or after the 2nd oestrus following sponge removal (Control group). Both groups were treated with either 10 mg of Lutalyse or 250 micrograms of Cloprostenol (Estrumate Coopers Animal Health Ltd.)

### **2.2.3 Blood Sampling and Progesterone Determination**

The luteolytic effect of the drugs was assessed by plasma progesterone levels in both parts of the experiment.

Blood sampling (as described in General Material and Methods of chapter 1) for plasma progesterone determination was carried for pregnancy diagnosis on d16, and to assess the pregnancy status before and after the luteolytic treatment, on the day of the treatment and during the next four to six days. Progesterone assays were conducted by ELISA (as described in General Material and Methods, Chapter 1).

### **2.2.4 Repetition of Treatments (Table 2.3)**

If, after two or more days of the 1st treatment with PGs, progesterone levels still remained luteal, the treatment was repeated with either Lutalyse or Estrumate until basal levels of progesterone indicating the completion of luteolysis had occurred.

Group	PMSG	Day of PG treatment	Lutalyse dose (ml)	treated n=	responsive to Lutalyse n=	RESISTANCE degree			
						1 n=	2 n=	3 n=	TOTAL n=
1	yes	13	2	6	*3	3	0	0	3
2	yes	16	2	2	0	3	0	0	3
			4	1	0				
3	yes	20	2	6	1	3	1	1	5
4	yes	28	2	5	1	2	1	1	4
5	yes	28	3	4	2	0	2	0	2
total (1-5)		16-28		18	4	8	4	2	14
6	yes	26 (+20)	2	3	0	0	1	2	3
7	yes	30 (+28)	2	4	3	0	0	1	1

\* unknown status of pregnancy (see text)

Table 2.1: Abortion or resistance to PGF-2a (Lutalyse) during different stages of early pregnancy (1:total resistance, 2: temporary and 3: partial).

Group	PMSG	day 20 treatment PG type	day 26 treatment PG type	treated n=	responsive to PG n=	RESISTANCE degree (1, 2 or 3)			TOTAL n=
						1 n=	2 n=	3 n=	
1	yes	Est	-	3	2	1	0	0	1
2	no	Est	-	4	3	1	0	0	1
3	yes	Lut	-	6	1	3	1	1	5
4	no	Lut	-	4	1	2	1	0	3
5*	yes	Lut	Est	2	2	0	0	0	0
6*	no	Lut	Est	2	2	0	0	0	0
7*	yes	Lut	Lut	3	0	0	1	2	3
8*	yes	Est	Lut	1	0	1	0	0	1
9*	no	Est	Lut	1	1	0	0	0	0
10*	no	Lut	Lut	1	1	0	0	0	0

\*Resistant ewes on day 20 (from groups 1-4) become groups 5-10.

Table 2.2. Type of Prostaglandin and its efficacy in inducing abortion following administration on day 20 and day 26 in both control and PMSG+ sheep.

Group	PMSG	Day 1st PG	Day 2nd PG	treated n=	responsive to PG n=	1 n=	2 n=	3 n=	resistance degree	resistance total
1	yes	13	26 E	3	3	-	-	-	-	0
2	yes	20	26 E	2	2	-	-	-	-	0
3	no	20	26 E	2	2	-	-	-	-	0
4	yes	20	26 L	4	0	1	1	2	-	4
5	no	20	26 L	2	2	-	-	-	-	0
6	yes	16	30 E	3	3	-	-	-	-	0
7	yes	20+26	30 E	1	1	-	-	-	-	0
8	yes	20+28	30 E	1	1	-	-	-	-	0
9	yes	20+26	30 L	1	1	-	-	-	-	0
10	yes	28	30 L	4	3	-	-	1	-	1

Table 2.3 Type of prostaglandin used (E: Estrumate vs L: Lutalyse) in a second injection on days 26 or 30 and its efficacy inducing abortion in ewes that resisted to a 1st injection with any of the PGs.



#### **2.2.5 Slaughterhouse material (Table 2.4)**

The tracts of the ewes that were treated with Lutalyse on days 28 and 30 were collected on day 34. These ewes had been run with a fertile ram from day 32 in order to detect postabortion oestruses. The number of follicles and corpora lutea were counted and their size estimates were recorded. The oviducts were flushed towards the fimbria to collect eggs or embryos. Uterus was opened and examined and the presence and number of foetuses was recorded.

### **2.3 Results**

#### **2.3.1 Classification of the responses to the treatment with prostaglandins.**

Different responses were observed after the administration of PGF<sub>2a</sub> or its analogue Cloprostenol. Ewes were classified according to whether or not they responded to the PG injections with total luteolysis, namely:

1) **SENSITIVE or RESPONSIVE** group, which responded with complete luteolysis and interruption of pregnancy, indicated by the fall of progesterone from luteal levels ( $> 4 \text{ nmol/l}$ ) to basal levels ( $< \text{or} = 3.5 \text{ nmol/l}$ ) within 24-144 hours of the PG administration (Tables 2.4 and 2.5)

	DAY 28		DAY 29	DAY 30		DAY 31	DAY 32	DAY 33	DAY 34 SLAUGHTER		
ewe #	Lut ml	P4	P4	Lut ml	P4	P4	P4	P4	old CL	new CL	Foetuses at postmortem
65	2	>32	18	2	32	-	2.8	-	0	1	0
184	2	>32	10	2	13	-	1.6	?	1	1	0
74	3	>32	3.8	2	2.7	-	1.6	-	2	0	0
188	3	>32	15	2	>32	-	3.9	3.6	8	0	5
29	3	>32	26	2	>32	-	7.7	8.6	5	0	4
91	2	>32	>32	0	-	>32	>32	>32	8	0	1

Table 2.4: Plasma progesterone levels (P4) following im injection(s) of Lutalyse (Lut) and the post mortem findings at day 34.

2) **RESISTANT** group which did not respond to the luteolytic effect of PGF2a, their progesterone levels remained luteal 4 to 6 days after the treatment with PGF2a. Three types of resistance were noted, namely:

(a) **TOTAL** : No sign of luteolysis was manifest, as progesterone levels remained unchanged after the PG treatment (Type 1).

(b) **TEMPORARY**: Progesterone levels decreased within 24 hours of the prostaglandin injection but then recovered within 2-3 days to the high pre-injection luteal levels (Type 2).

(c) **PARTIAL**: Progesterone decreased to lower luteal levels after the prostaglandin injection and stayed this way for the sampling period (Type 3).

**2.3.2 Resistance to PGF2a (Lutalyse) in superovulated ewes during early pregnancy (d13 to d28-30). (Table 2.1 and graph 2.1a)**

#### **Dose effect**

Although the number of ewes compared was very small, the use of the double dose (4 ml) in one ewe did not change the results of the treatment on day 16, and all these ewes (n=3) presented total resistance with both 2ml and 4 ml. For later considerations, these sheep will be regarded as one group.

On day 28, more resistance was found with a lower dose (2 ml vs 3 ml). A higher proportion of ewes presented resistance with the lower dose (4/5 vs 2/4) and more of the resistant ewes presented total resistance (2/4 vs 0/2). (Graph 2.1a).

### **Stage of the pregnancy (groups 1 to 5)**

Group 1: On day 13, the progesterone levels of 3 ewes out of 6 treated with Lutalyse, returned to basal levels 24-144 hrs after the PG injection. At this early stage it was not possible to know the pregnancy status at the PG injection by their progesterone levels. For that reason, the luteolysis observed after PG within the normal 15-19 days could have been either spontaneous (if they were not pregnant) or induced by the PG injection. The other 3 ewes treated on day 13, maintained their progesterone levels luteal and unchanged ( $>30$  nmol/l) after the PG injection, at least until day 26. In retrospect, they were assumed to have been pregnant when injected with PG and therefore they presented total resistance of the pregnancy CL to luteolysis.

Groups 2-5: Resistance of the CL to 2, 3, and 4 ml of Lutalyse was found in the majority of the ewes that were known to be pregnant (14/18, 78 %) on days 16, 20 and 28. Considering only the ewes treated with 2 ml, resistance to luteolysis was found in (11/13, 85%) on days 16, 20 and 28 of pregnancy.

When the different days of treatment were compared using 2 ml of Lutalyse, it was found that the incidence of resistance decreased and the type of resistance changed as the pregnancy advanced (shown in graph 2.1a). The percentage of resistant ewes was decreasing towards day 20 to 28 and the most common patterns of resistance shifted from being total resistance to become temporal sensitivity and partial resistance.

### **Effect of 1 or 2 administrations (groups 6 and 7):**

All the ewes which were resistant on day 20 were given a second injection one week later (day 26) and again showed resistance to the second injection (3/3).

Therefore the single treatment on day 28 gave similar percentage of resistant ewes (4/5, 80%) when compared with the double treatment on days 20 and 26 (5/6, 83%). However, when a second injection was given on day 30 to ewes which showed resistance to Lutalyse on day 28, only 1 out of 4 was resistant to luteolysis.

### **2.3.3 Estrumate versus Lutalyse and the effect of the numbers of Embryos and/or Corpora Lutea (PMSG effect). (Table 2.2)**

#### **Estrumate versus Lutalyse on day 20 (groups 1 to 4)**

A similar proportion of superovulated and control ewes were resistant to both Estrumate (1/3 vs 1/4) and Lutalyse (5/6 vs 3/4) respectively on day 20. Because there was no significant difference, the groups were joined together in order to compare Estrumate with Lutalyse. Estrumate caused luteolysis in a higher proportion of ewes (5/7) on day 20 than Lutalyse (2/10).

#### **Estrumate versus Lutalyse on day 26 with pretreatment on day 20 (groups 5 to 10)**

All the ewes that were resistant to Lutalyse on day 20, both the superovulated and the controls, responded with luteolysis to Estrumate on day 26 (4/4). In contrast, only 2 out of 6 were sensitive and responded to Lutalyse on day 26. These two ewes belonged to the control group.

#### **Estrumate versus Lutalyse on days 26 or 30 after various days and PG types of pretreatment (Table 2.3)**

All the ewes (12/12) that were retreated with Estrumate on days 26 and 30 completed luteolysis whereas only 6/11 did so when retreated with Lutalyse on those days.

The second injection of Estrumate (days 26 and 30) was luteolytic in all the cases of the present experiment

independently of different circumstances, namely :1) Whether or not the ewes were superovulated, 2) The stage of the pregnancy at the 1st (13, 16, 20) or second treatment (26 or 30) and 3) Whether the interval between both injections was 1 week or more (6 or 14 days).

The sensitivity to a second injection of Lutalyse on days 26 and 30 was associated with a series of circumstances:

1) Superovulation with PMSG. Only control sheep were sensitive on day 26 (2/2) and all the superovulated sheep were resistant (4/4) (groups 4 and 5).

2) More ewes responded later (on day 30) and two or four days apart from the first injection than when it was given earlier (on day 26) and 6 days apart (4/5 vs 2/6). (groups 4+5 vs 9+10).

#### **2.3.4 Estrumate versus Lutalyse: speed of the Luteolysis (Tables 2.5 and 2.6).**

In the non resistant sheep, luteolysis was completed faster with Estrumate than with Lutalyse. Most of the ewes (15/18) exhibited basal levels <sup>of progesterone</sup> (< or = 3.5 nmol/l) within 48 hours of the injection with Estrumate, whereas with Lutalyse, only half (7/14) reached basal levels by 48 hours post-injection.

#### **2.3.5 Post-mortem findings of the reproductive tracts (Table 2.4)**

Three out of four ewes sensitive to the second injection of Lutalyse on day 30, showed very low basal levels of progesterone (1.6 and 2.8 nmol/l) two days after the injection. Four days after the injection, the examination of their tracts collected at the slaughter-

house, revealed no fetuses present and presence of recently formed corpora lutea (red or pink).

The other sensitive ewe (#188) exhibited basal but higher levels of progesterone (3.9 and 3.6 nmol/l) than the former ewes, two and three days after the prostaglandin injection. Four days post-injection, fetuses accompanying mature corpora lutea were found and young CL were absent in this ewe.

Within the group of ewes resistant to the first (day 28) or both injections (28 and 30), progesterone remained luteal until day 33. On day 34 (day of slaughtering) the tracts revealed presence of fetuses and mature Corpora Lutea. More Corpora Lutea (5 and 8) than fetuses (4 and 1 respectively) were observed.

			Hours following PG treatment (P4 nmol/l)			
Day of treatment	PMSG	Prev PG (day)	0	24	48	72 or 144
20	yes	-	>32	-	4.4	1.9
	yes	-	10.3	-	3.5	(144h) -
20	no	-	>32	5	1.9	-
	no	-	>32	7	1.9	-
	no	-	>32	4	2.1	-
26	yes	Lut d13	>32	-	2.8	-
	yes	-	>32	-	3.3	-
	yes	-	>32	-	2.5	-
30	yes	Lut d16	>32	-	2.3	-
	yes	-	>32	-	2.3	-
	yes	-	>32	-	2.1	-
26	yes	Lut d20	23.5	3.3	2.2	-
	yes	-	>32	>32	6.6	4.5 (72h)
26	no	Lut d20	>30	-	2.6	-
	no	-	16.5	-	1.6	-
30	yes	Lut d20&26	>32	-	4.0	-
39	yes	Lut d20&28	15.2	-	1.9	-
39	yes	Lut d28	>32	-	3.4	- -

Table 2.5 Daily P4 levels in ewes which responded to Estrumate administration, some having been previously treated with PG.



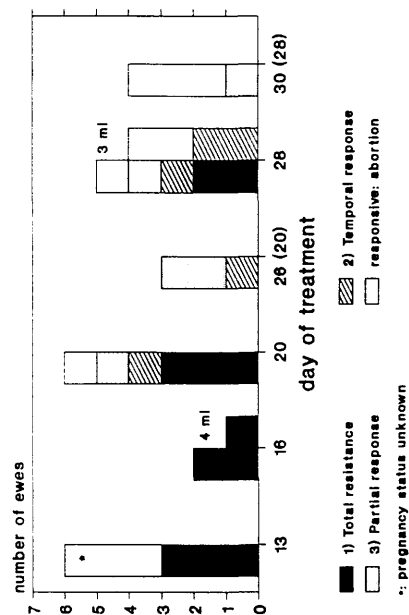
			Hours following PG treatment (P4 nmol/l)					
day of treat	PMSG	prev PG (day)	0	24	48	72	96	144
13	yes	-	>32	>32	10.4	6.8	4.2	5.7
	yes	-	>32	29	6.7	-	-	2.7
	yes	-	11	4.7	1.9	1.6	1.6	3.1
20	yes	-	>32	-	5.7	-	-	2.6
	no	-	21.1	6.7	2	-	-	-
26	no	Lut d20	>32	-	15.2	12.7	1.9	-
	no	Est d20	13.8	-	11.9	8.6	1.5	-
28	yes	-	>32	3.8	2.7	-	1.6	-
	yes	-	>32	3.8	3.5	-	1.6	-
	yes	-	>32	8.9	4.7	-	1.6	-
30	yes	Est d20 Lut d26	>32	-	3.5	-	-	-
	yes	Lut d28	>32	-	2.8	-	-	-
	yes	"	13	-	1.6	-	-	-
	yes	"	>32	-	3.9	3.6	-	-

Table 2.6 Daily P4 levels in ewes which responded to Lutalyse administration, some having been previously treated with PG.

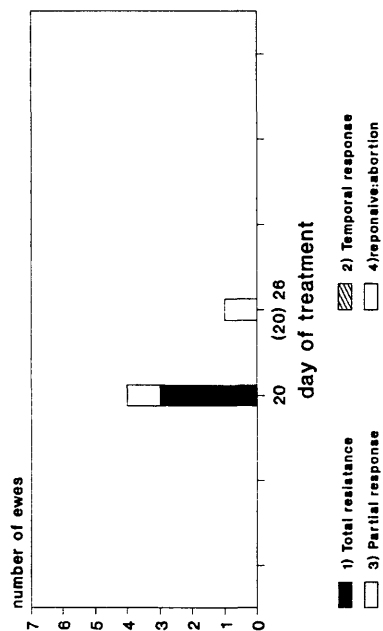
Treatment details			Reference	% sheep resistant to the luteolytic effect of PG and oestradiol during pregnancy (day of treatment)				
Drug	Dose	PMSG		day 19 or 20	day 26	day 28	day 30	day 33
PGF2a	4mg	no	Silvia, 1986	partial	absent	-	absent	-
PGF2a	10mg	no	Lacroix (1986)	16%	-	-	-	-
PGF2a + Indomethacine	10mg	no	Lacroix (1986)	25%	-	-	-	-
Cloprostenol	250ug	1500iu	Present work	33.4%	absent	absent	absent	absent
Cloprostenol	100ug	0,100 400 & 700iu	Nancarrow (1982)	65%	yes	absent	-	-
PGF2a	10mg	1500iu	Present work	83.3%	100%	80%	25%	-
Oestradiol	125ug	no	Lacroix (1986)	100%	-	-	-	80%

Table 2.7 Resistance of the corpus luteum to luteolysis after day 20 of pregnancy.

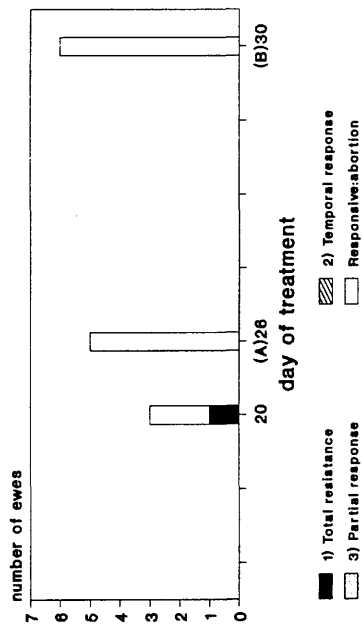
### 2.1a Resistance to Lutalyse between d13 and d30 (PMSG+)



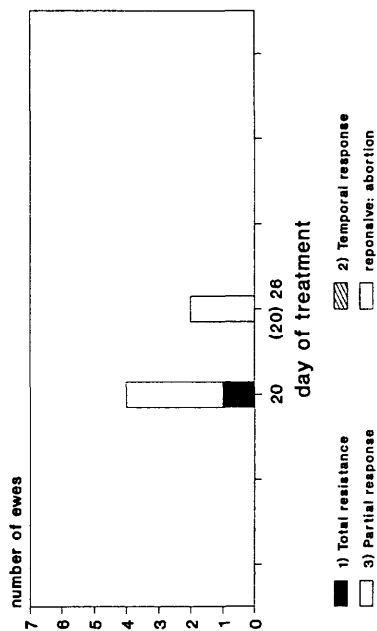
### 2.1b Lutalyse day d20 and 26 (control)



### 2.1c Cloprostenol on day 20, 26 and 30 (PMSG+)



### 2.1d Cloprostenol day d20 and 26 (control)



A: 13 or 20  
B: 16, 20-26 or 20-28

Graph 2.1 Resistance to Lutalyse and Estrumate at different stages of gestation

## 2.4 Discussion

### 2.4.1 The pattern of the effect of Lutalyse administered at different stages of pregnancy in superovulated ewes.

A high percentage of superovulated pregnant ewes (14/18 or 78%) were resistant to the luteolytic treatment of 10, 15 and 20 mg of Lutalyse on days 16, 20 and 28. The smaller dose of Lutalyse mainly used in the present experiment (10 mg) has been demonstrated to be luteolytic in at least 60% of cyclic ewes treated in their luteal phase (Douglas & Ginther, 1973; Hawk, 1973; Hackett & Robertson, 1980; Hoppe & Slyter, 1989). The number of corpora lutea has been found to be associated with a greater degree of resistance only in pregnant sheep and not in cyclic ones (Silvia & Niswender, 1984; Nancarrow et al., 1982). Therefore, although it was not possible to assess the number of embryos and CL and to correlate them with the resistance observed, this does not seem to be attributable to the presence of multiple corpora lutea induced by PMSG, but to the presence of multiple embryos. Therefore, the resistance to luteolysis found in the present study, has to be attributed to the antiluteolytic effect of the conceptus/ses. Resistance of pregnancy to Lutalyse on day 13 was demonstrated in at least 3/6 ewes in the present experiment, which may be 50 to 100% depending of whether the other 3 sheep that underwent luteolysis were pregnant before the PG treatment or not.

In the present experiment, the percentage of treated ewes that presented resistance to 2 ml of Lutalyse decreased from the earlier stages of the pregnancy towards the end of the first month, 100%, 83.4% and 80% of resistant ewes were found at days 16, 20 and 28 respectively. The finding of total and temporal resistance on days 20 and 28 in most ewes, differs from

the transitory character described previously for the pattern of resistance of the CL to PGF2a (Silvia & Niswender, 1986), limited to the days around maternal recognition (day 13) and preattachment (i. e. before day 16). Only on those days the concentrations of progesterone were restored to pretreatment levels (i.e. total or temporal resistance of the present work) by 36 hours in most ewes. Luteal responsiveness to PGF2a returned on day 19 or day 20 in most cases and by day 26 of pregnancy, resistance could no longer be demonstrated (Silvia & Niswender, 1986). The transitory character of the resistance to PGF2a has been found to parallel the pattern of secretion of the embryonic antiluteolysin ovine trophoblast protein one (oTP-1) (Godkin et al., 1982; Guillomot, Michel, C., Gaye, Charlier, Trojan, and Martal, 1990), presumably responsible for that resistance because of the antiluteolytic effect demonstrated during this period (Godkin, Bazer, Thatcher, and Roberts, 1984; Vallet, Bazer, Fliss and Thatcher, 1988). The secretion of this protein (oTP1) has been reported to be maximal midway between days 13 and 16 (Godkin et al., 1982), to decline by day 21 and cease on day 23. This has been reported by a number of authors using different techniques (Godkin et al., 1982; Hansen, Imakawa, Polites, Marotti, Anthony and Roberts, 1988; Farin, Imakawa, and Roberts, 1989). Recent studies on the oTP1 mRNA also confirm the above transitory pattern, the arrest of expression occurring in the regions of the trophoblast which have stabilised cellular contacts with the uterine epithelium during the implantation process which starts in the ewe on day 17 (Thorburn & Rice, 1990; Guillomot et al., 1990).

However a more spread pattern of secretion for both trophoblastic interferons (tINF) in the ewe (OTP-1) and the cow (bTP-1) have been recently suggested by a group of workers. A second period of secretion for OTP-1 has been found between days 25 and 45 (Bazer, Thatcher,

Hansen, Mirando, Ott, and Plante, 1991) in the ewe. In the cow , bTP-1 is also secreted during the postattachment period (days 24-38) (Godkin, Lifsey, and Gillespie, 1988). The antiluteolytic activity detected at the postattachment period in the present experiment may be explained by either a long lasting effect of the first period of secretion of oTP-1 or the possible antiluteolytic effect of the second period. As far as the author is aware, the second possibility has not yet been tested experimentally. The other alternative is that other agents that have been proposed as luteotrophic may be involved in the postattachment period (after day 17).

Some authors have found that although the secretion of oTP-1 terminates in the periattachment period (d16-d21), the antiluteolytic effect of the conceptus or oTP-1 itself may last until attachment is completed on day 28 (King et al., 1982) and in some cases until days 50-60. Conceptus removal on days 21-23 (Martal et al., 1979) and after day 20 (McCracken et al., 1984), ie after the maximal secretion in the 1st period of oTP-1 and before the 2nd period, was associated with extension of the CL lifespan beyond day 50. This indicates a long lasting antiluteolytic effect of the conceptus. Furthermore, the use of PMSG may have prolonged the "luteoprotection and/or antiluteolytic activity" in the present work further than Silvia & Niswender (1986), as luteal levels of progesterone seem to be maintained for a longer time with the removal of two embryos rather than one (McCracken et al., 1984). oTP-1 can be found in the uterine lumen at least until day 24 (Kazemi, Malathy, Keisler and Roberts, 1988) indicating that it may persist in the uterus, where it is thought to exert its function. Its administration into the uterus of cyclic ewes has caused maintenance of the CL for 21, 25, 37 , 34 and more than 52 days at least in one occasion (Godkin et al., 1984; Vallet et al., 1988).

oTP-1 secretion measured by RIA at the 2nd period, was greatest on day 25 (162 +95 ng/ml by RIA) and much lower levels, about 1 ng/ml, were found beyond that day (Ott, Mirando, Davis, Fliss and Bazer, 1989). This peak of oTP-1 secretion on day 25 may also explain the present finding of a high proportion of superovulated ewes resistant to Lutalyse observed at the 2nd injection on day 26 (3/3), when compared with day 20 (5/6) or day 28 (4/5), although only temporal and partial responses to Lutalyse were found on day 26 in superovulated sheep. The fact that the slower luteolysis (>72 hrs) observed with Estrumate in a single animal occurred also on day 26, may support the supposition that resistance may peak on days 25-26. The direct comparison of day 20 and day 26 was not planned, but it appears that the two peaks of resistance found here on days 16 and 26, coincide with the peaks of oTP-1 observed on days 16 (Godkin et al., 1982) and 25 (Ott et al., 1989), which may or may not be a significant observation because of the small number of animals treated. In the present work, resistance of the CL to Lutalyse was found on days 26, 28 and 30 of pregnancy, which coincides with the 2nd period of secretion of the oTP-1. Despite the extensive literature that describes a transitory pattern of both oTP-1 secretion and resistance, ending on day 22, there are various contradictory reports of resistance being found later in the pregnancy to various luteolytic drugs including PGF<sub>2a</sub>, PGF<sub>2a</sub> analogue and Estradiol-17 $\beta$  (Nancarrow et al., 1982; Lacroix & Kann, 1986) which are consistent with the present findings.

Ott et al. (1989) suggested that the second period of secretion of oTP-1 remains low, but detectable, on days 40 and 45 (quoted by Bazer, et al., 1991) and undetectable after day 60 in chorioallantoic explants. The cessation in the detection of this protein between days 45 and 60 coincides with the time in which the CL is no longer indispensable in the ewe for the

production of progesterone and the maintenance of pregnancy (Casida & Warwick, 1945; Denamur & Martinet, 1955). This supports the idea that oTP-1 may be antiluteolytic also at this stage, possibly in order to maintain the CL on which the pregnancy maintenance depends. Until the placenta can produce progesterone, the role of oTP-1 may be to ensure the maternal supply of progesterone by the CL. In the goat, which is CL dependant throughout pregnancy, resistance to PGF2a has been suggested on day 52 to 63 (Bretzlaff, Weston, Hixon, and Ott, 1988). If this antiluteolytic activity can be more strongly demonstrated and further studies of the cTP-1 or other antiluteolytic substances carried out, the maintenance of the CL after the attachment may be better understood. If there is a similar mechanism in the goat as in the sheep, it would be expected that caprine trophoblast protein-one (cTP-1) would have to be secreted throughout pregnancy in order to protect the CL, as it is essential until parturition.

Reports on the resistance to luteolytic drugs (PGF2a, PGF2a analogue and Oestradiol-17B) between days 19 and 33, together with the present findings with Lutalyse and Estrumate after day 20, vary considerably. Different combinations of luteolytic drugs and doses were used in the presence of different numbers of corpora lutea. Resistance to PGF2a has been suggested to be dose-dependant (Silvia & Niswender, 1984) and also related to the number of embryos present, which has been assumed to be closely related with the number of CL (Nancarrow et al., 1982; Silvia & Niswender, 1984; Silvia et al., 1984a). Comparison of various reports on resistance from day 20 are shown in table 2.7. The different combinations of drugs used are classified according to the luteolytic efficiency shown on day 20. The higher the percentage of resistant sheep found on day 20 with any given treatment (luteolytic drug/dose/use of PMSG), the more likely it is that resistance will be found later in the pregnancy. This



shows that the treatments which fail to terminate pregnancy at day 20 are also less efficient at later stages. The resistance during the second period of oTP-1 production appears to be lower than during the 1st period (d13-d21). This could explain both the failure of its detection by Silvia & Niswender (1986) and the present results with the more potent drug Estrumate. In both cases the dose may have been sufficiently large to overcome the resistance produced by the number of embryos present, as indicated by the small % of unresponsive ewes found on day 20. Silvia & Niswender (1986) was not looking at complete luteolysis but suppression of progesterone production. It may be possible that the smaller resistance suspected in the second period may be correlated with production of smaller quantities of oTP-1. The differences between oTP-1 seem to be smaller on day 25 (Ott et al., 1989) than on day 16 (Ashworth & Bazer, 1989), but a detailed description of the levels of oTP-1 found in the second period of secretion is still not published and direct comparison therefore can not be made.

#### **2.4.2 Comparison of Estrumate with Lutalyse.**

Despite the small number of animals used in the present experiment, there are several findings that suggest the superiority of Estrumate in producing luteolysis and pregnancy termination compared with Lutalyse at the dosages used.

Comparison of both drugs on day 20 showed a higher proportion of ewes that responded to Estrumate than to Lutalyse (5/7 vs 2/10). Estrumate was also superior, in terms of inducing luteolysis and abortion in superovulated ewes, on day 20 (2/3) than Lutalyse on day 28 (3/9). It would be expected that the CL would be more sensitive on day 28 than on day 20 as resistance to Lutalyse was observed to decrease as the pregnancy advances in this work and the main period of oTP1

secretion finishes by day 21 (Godkin et al., 1982). Therefore Estrumate seems to be a more potent luteolytic drug than Lutalyse, as it produces luteolysis in a higher proportion of ewes than Lutalyse under either the same conditions of luteoprotection (both on day 20) or even with less luteoprotection (day 28 Lutalyse and day 20 Estrumate).

Further evidence for a difference in potency of the two Prostaglandins is found in the speed of luteolysis in those ewes which responded. The rate of luteolysis has been reported to be slower in pregnant ewes than in cyclic ewes (24 hrs of difference) using both Lutalyse (Lacroix & Kann, 1986) and Estrumate (Nancarrow et al., 1982). This has also been observed in superovulated heifers when treated with PGF<sub>2a</sub> (Kosugiyama, Britt and Hafs, 1978) and it may reflect the resistance of the pregnancy CL to luteolysis. Most of the ewes (15/18) in the present work, treated with Cloprostenol on different days of pregnancy, experienced rapid luteolysis and their plasma progesterone fell to basal levels within 48 hours of the treatment. Using Lutalyse, only 7/14 reached basal levels within 48 hours and the remainder responded within 72-144 hours. Direct comparison of both drugs is not available in the literature, but the Estrumate figure of the present work is in line with previous reports in the literature, basal levels of progesterone were reached by 24 hours (Nancarrow et al., 1982) and the lowest levels by 48 hours (Nancarrow et al., 1982, Tyrrel, 1981). Lutalyse produced a slower luteolysis in some cases of the present study compared with previous work (Lacroix & Kann, 1986), who reported 72 hours for pregnant ewes using the same dose but divided into four and given twice for two days (19 and 20).

#### **2.4.3 Dose**

It is not clear whether the doses used here are comparable or if there is any difference in the potency or mechanism of action of Estrumate which make it less vulnerable to the antiluteolytic effect of the early pregnancy CL.

##### **a) Dose of Cloprostenol and PMSG**

Nancarrow et al.(1982), using smaller doses of both PMSG (0, 100, 400 and 700 I.U.) and Cloprostenol (ICI, Australia) found more resistance on day 21 when only 8/23 (35%) ewes responded with luteolysis, whereas in the present experiment 5/7 (71.4%) responded. He also found that the refractory sheep were the ones that had higher ovulation rates (3.2 vs 1.8). In contrast, in the present experiment, there was no difference between PMSG treated (2/3) and Control (3/4) ewes which responded with luteolysis. This may suggest that the higher dose of Cloprostenol used here (250 µg) was probably sufficient to cause luteolysis in most ewes regardless of their presumably high ovulation rates. However it must be said that the PMSG ovulatory response was unknown in the present work, and this response can vary with the individuals and the batch of the drug (Smith, 1988).

It may be possible however, that Nancarrow et al.'s dose of Cloprostenol (100 µg) was too low to produce luteolysis with the higher ovulation rates induced by PMSG. Further evidence supporting this view is found in the work of Greyling and van der Westhuisen (1979) who reported temporary response even in cycling ewes with a slightly larger dose than that used by Nancarrow et al. (125 µg vs 100 µg). In addition, Reid & Crothers (1980) also found resistance to 125 µg on days 22 and 23 of pregnancy in ewes that did not receive PMSG to stimulate ovulation.

However, Nancarrow (1979) aborted 6 out of 6 non-superovulated ewes, using 125  $\mu$ g of the synthetic PG on days 19 to 21, indicating that there is probably no resistance to this dose when PMSG is not used. Tyrrell et al. (1981) found no differences between 125 or 250  $\mu$ g of Cloprostenol (Estrumate, ICI Australia Ltd) in non-PMSG treated sheep, which may further indicate that in the present experiment the higher dose (250  $\mu$ g) may have compensated for the possible presence of multiple conceptuses induced by superovulation with PMSG. The higher dose used in the present experiment could therefore explain the smaller resistance to Cloprostenol found on day 20 in the present experiment in comparison with Nancarrow et al.'s findings on day 21.

It therefore appears that Estrumate at the dose of 250  $\mu$ l is a fairly effective drug to induce abortion on day 20 in ewes treated with 1500 I.U. of PMSG (66.6%). In non-superovulated sheep, 3 out of 4 responded to that dose, but the suggested lower dose of 125  $\mu$ l (Tyrrell, 1981) may or may not be (Reith and Crothers, 1980) enough, this has to be confirmed.

#### **b) Dose of Lutalyse**

The number of ewes responding to Lutalyse (2/10) on day 20 in the present experiment, is much smaller than 5/6 reported by Lacroix & Kann (1986) using the same dose but divided into four and given twice for two days (19 and 20). He was not using PMSG, but even when only the present figures of the Control ewes are compared (1/6 vs 5/6), less ewes responded in the present work to Lutalyse. He reported that when the source of endogenous PG was restricted by treating with Indomethacine (a PG synthesis inhibitor) the resistance was greater (6/8), indicating that the resistance to PGF<sub>2a</sub> is dose-dependant. This last figure is more similar to the present findings (8/10), which may

indicate that a higher dose was necessary in the present work or that the division of the dose makes it more effective in causing abortion. This latter point will be discussed later.

Nancarrow et al. (1982) found a higher number of embryos among those ewes that were refractory to 100  $\mu$ g of Cloprostenol, concluding that the embryonic antiluteolytic effect is quantitatively related to the number of embryos present. Silvia & Niswender (1984) found positive correlation between the minimum luteolytic dose required for pregnant ewes with the number of CL present. It was shown in that work that 10 mg of PGF2a, the dose used here, is the minimum luteolytic dose for pregnant ewes with 2 corpora lutea, this being higher than the dose required to cause luteolysis of a single CL (6 mg). In the present experiment, more than 2 CL and embryos could be expected in the ewes that responded to the PMSG treatment, according to Cameron (personal communication, 1987). Therefore the dose of Lutalyse most commonly employed in the present work (2 ml = 10 mg) could have been too low to produce luteolysis when more than 2 embryos were presumably present. If the dose of Lutalyse was small to terminate pregnancy with multiple embryos, this could have contributed to the high resistance observed to this drug (2/10) in contrast with Estrumate (5/7) on day 20.

Higher doses of Lutalyse (3 ml and 4 ml) were used in number of ewes with inconclusive results due to the small number of animals. The use of 4 ml in a single ewe on day 16 gave total resistance as was found in the ewes (n=2) treated with just 2 ml. This may be an individual response or may indicate that even 4 ml was not a luteolytic dose considering the high degree of resistance found at day 16 in the present experiment. This is in agreement with Silvia & Niswender (1986) who found that resistance decreased from day 16 coincident

with the decrease of the oTP1 after day 14 (Guillomot et al., 1990). The resistance on day 16 in the present experiment, could have been further increased by the presence of presumably multiple embryos in the uterus. The use of 3 ml on day 28 when less resistance than on day 20 was observed, did not change the results dramatically when compared with 2 ml, but it did gave slightly better results in terms of less ewes showing resistance (2/4 vs 4/5) and the fact that none of the resistant sheep exhibited the maximum type (0 vs 2).

Differences between Estrumate and Lutalyse were observed in the control ewes. Despite the very small number of ewes that were compared, it appears that more resistance was found with 2 ml of Lutalyse (3/4) than with 1 ml of Estrumate (1/4). This may further support the hypothesis that 2 ml of Lutalyse was a small dose insufficient to produce luteolysis even within the control ewes (probably with 1 to 3 CL) whereas 1 ml of Estrumate (250  $\mu$ g) seemed to be enough to produce luteolysis in most of the PMSG treated sheep (2/3) and controls (3/4). If the doses of both PGs used here are compared with the ones recommended for cattle after dose titration studies (Schultz, 1984), the dose of Lutalyse in the present experiment (10 mg) was lower than half the effective dose for cattle (25 mg/head), whereas 250  $\mu$ g is higher than 1/2 the dose found for cattle (375  $\mu$ g/head). Some studies on the use of PGF2a to synchronize oestrus indicated that 15 and 20 mg were more recommended for this purpose (Hackett & Robertson, 1980; Henderson, Downing, Beck and Lees, 1984), which may suggest that the luteolytic dose for ewes is higher than the used here, even for cyclic ewes.

#### **2.4.4 The effect of second administration of prostaglandins**

All the ewes (Control and PMSG+) (n=13) which were resistant to PGs at any stage of pregnancy responded with luteolysis when they were retreated with Estrumate

at various stages (days 26, 30 and 39). In contrast using Lutalyse (on days 26 and 30), resistance to the second injection was observed in most superovulated ewes. Comparing only similar stages of the pregnancy, Estrumate was also more effective (on days 26 and 30) than Lutalyse , when refractory sheep to either of the PGs (on different days) were retreated with one or the other drug (12/12 vs 5/11).

The differences in response found with the second injections of the two types of PG in the present work, may also support the hypothesis proposed earlier that Estrumate is a more potent luteolytic drug than Lutalyse after day 20 at the doses employed here. The results of the present work agree with Nancarrow et al's proposal (1982) that when multiple embryos cause resistance on day 20 to Estrumate, a second injection a week later may terminate pregnancy. The 2nd injection may work because of the more advanced stage of the pregnancy or because the 1st injection may have an effect of priming. In the present experiment, these cannot be differentiated for Estrumate, because all the sheep treated after day 20 had been previously treated. A second injection on day 26 with Lutalyse in the present experiment failed to terminate pregnancy in more superovulated animals than on day 28 alone (3/3 vs 4/5). Therefore there was no indication that pretreatment on day 20 increased the effectiveness of the producing abortion of the injection on day 26 than on day 28 alone, although the number of ewes treated was very small and precisely the same days are not compared. Slightly better results were achieved in the PMSG+ group (3/4 ewes aborted) when the second injection with Lutalyse was given on day 30, just two days after the first. Assuming that priming has no effect, the present results show that resistance to both PGs seem to be transitory and the resistance to Estrumate appears to decline more rapidly than with Lutalyse. If there was a difference in the effect that

the first injection may have had, ie synergistic effect on Estrumate but not in Lutalyse, this would have implications on the mechanism of action of both drugs. To induce abortion, whatever the mechanisms acting here, Estrumate proved to be more useful. Therefore if resistance is observed after the first treatment in some of the sheep, those animals could be given another injection after day 26 which seems to be 100 % effective at the dose of the present experiment.

According to the present results it would be advisable to use Estrumate rather than Lutalyse to terminate pregnancy, using 250  $\mu$ g. When the day of mating is known, a small degree of resistance was found on day 20 and therefore a later stage would be recommended. Although this was not studied with Estrumate, the results with a single injection of Lutalyse together with the repetition of treatments on days 26 and 30 indicate that resistance declines with the advancement of pregnancy and therefore day 30 would be more recommended. The most common indication for abortion in early pregnancy however is mismating in a flock of ewe lambs when a ram escapes. The multiple stages of pregnancy which can be expected are usually unknown and this is essential for the success of the treatment, as the CL responsiveness changes along the pregnancy. The periods to avoid would be a first period of refractoriness between the day of mating and day 4 (Acritopoulou & Haresign, 1980), and a second one with reduced sensitivity between days 12 to 21 (Silvia & Niswender, 1984a) or even day 26, according to Nancarrow et al. (1982) and the present results. In the other hand if the ram has been mating ewes for three weeks it may be too late to wait for a month as some of the ewes would be on day 50, when the placenta can maintain pregnancy on its own.



Therefore, a first injection of Estrumate given in a flock where the stages of pregnancy are unknown will probably terminate pregnancy between days 4 and 11 and from 25 to 50 days. A second injection can be given on 8 days later, ewes that were at metoestrus in the forming period at the first injection will be now in a responsive period (days 8-12) and ewes that were refractory in early pregnancy (day 12 to 25 or later) will be now on day 20 and later. In the present work both control and superovulated ewes were still resistant to Estrumate on day 20 (2/7) therefore a 3rd injection may be necessary again 10 days later, this injection may also terminate pregnancy in those ewes that were on refractory to the second injection on day 12. Further information is needed about the sensitivity of the CL to Estrumate from day 20 onwards, in order to avoid the 3rd injection.

## CHAPTER 3

### STUDIES ON THE EFFECT OF PGE<sub>2</sub> ON THE CERVIX AND VAGINA

#### 3.1 INTRODUCTION

The human cervix must undergo a great metamorphosis in late pregnancy and labour to allow the passage of the newborn without injury. In the last trimester, the cervix softens and during prelabour, namely the last four weeks of pregnancy, further softening occurs with effacement (thinning of the cervical wall and shortening of the canal). As labour proceeds, dilatation of the lumen occurs. The whole process is known as "cervical ripening" (reviewed by Danforth, 1983) and the failure of the cervix to "ripen" at labour is a pressing problem for obstetricians whereas, on the other hand, dilatation can occur too early with preterm delivery (Calder, 1981). The control of the cervical ripening is therefore of interest in human obstetrics and much research has been carried out in this area.

The basic structure of the human cervix is connective tissue (Danforth, 1947) and it is believed to play a major role in both dilatation and closure of the cervix. The changes that occur in late pregnancy and labour in the connective tissue have been investigated by microscopy (Danforth, 1960; Junqueira, Zugaib, Montes, Toledo, Krisztan and Shigihara, 1980; Minamoto, Arai, Hirakawa and Nagai, 1987; Yoshida & Manabe, 1990) and biochemistry. Ultrastructural studies have shown that cervical ripening involves disintegration of collagen bundles, increase in the amount of ground substance and the presence of white cells (Junqueira et al., 1980; Liggins, 1981; Minamoto et al., 1987). The changes in the physical properties of the cervix have been related to quantitative and qualitative changes in two components of the connective tissue, collagen and

proteoglycans (reviewed by Hillier, 1990), but the biochemical mechanisms involved are not fully understood. It is now well established however, that exogenous prostaglandin PGE<sub>2</sub> or its analogues induce cervical ripening early in pregnancy, at term and in nonpregnant women (Lueken & Lindermann, 1979; Ferhmann, 1983; Calder, 1990; Calder & Greer, 1990; Hillier, 1990). PGE<sub>2</sub>-induced cervical ripening at term, seem to mimic the changes of spontaneous ripening both histologically (Uldbjerg, Ekman, Malmström, Sporrang, Ulmsten and Wingerup, 1981) and biochemically (Ekman, Malmström, Uldbjerg, and Ulmsten, 1986). Although PGE<sub>2</sub> and its analogue, Sulprostone, can also produce cervical ripening in the first trimester when used for termination of pregnancy (Rath, Adelman-Grill, Pieper and Kuhn, 1987a; Rath, Adelman-Grill, Schauer and Kuhn, 1987b; Fehrmann & Praetorius, 1983) and similar histological changes are observed (Theobald, Rath, Kühnle and Kuhn, 1982), biochemical studies (Rath et al., 1987a and 1987b) indicate that this PG-induced cervical ripening may differ in its mechanisms from the physiological cervical ripening found at term (Hillier, 1990).

The clinical use of prostaglandins, specially PGE<sub>2</sub>, is widespread in humans to treat unfavourable cervix at term or to induce therapeutic abortion in the first trimester (Calder, 1990). The advocates of this method claim that the safety and reliability of it are based on the physiological action that prostaglandins, especially PGF<sub>2</sub>a and PGE<sub>2</sub>, have on the onset and progression of labour in man (Calder & Greer, 1990; Challis and Olson, 1988). The first PG available for induction of labour was PGF<sub>2</sub>a, but this was later replaced by PGE<sub>2</sub>. The uterotonic potency of PGE<sub>2</sub> that is ten times greater than that of PGF<sub>2</sub>a allows the use of much smaller doses and diminishes the possible secondary effects. PGE<sub>2</sub> is superior to PGF<sub>2</sub>a in inducing labour in terms of potency, specificity and

toxicity (Calder, 1990). Keirse (1990) who analyzed studies done in man that directly compared PGE<sub>2</sub> with PGF<sub>2a</sub> effects, found an advantage for the smaller dose of PGE<sub>2</sub> (5 mg) against the greater dose of PGF<sub>2a</sub> (25 mg or 40 mg). PGE<sub>1</sub> had also been tried but the results did not differ from PGE<sub>2</sub> which has been much more extensively studied.

The different routes of prostaglandin administration have been reviewed by MacKenzie (1981), Keirse (1990) and Calder (1990). There are differences between routes in terms of efficacy, safety and economy of dosage. Local routes are preferred to general ones (oral and intravenous) because they are considered more successful and smaller doses are required. They also minimize side effects that can occur when PGE<sub>2</sub> is absorbed systemically as this PG also may act on the gastrointestinal tract producing nausea, vomiting and diarrhoea. The local routes that are applicable for cervical ripening are namely extra-amniotic, vaginal and endocervical. There is a compromise in the selection of one of these local routes between the simplicity, safety and non-invasiveness compared with the economy of the dosage, less side effects, efficacy and more specific delivery. The extra-amniotic route consists of the aseptic introduction of a catheter through the cervix so as to inject the PGs into the space between the fetal membranes and the uterine wall (Wiqvist & Bygdeman, 1970; Calder Embrey, Tait & Embrey, 1977; Miller, Calder and Macnaughton, 1972). This route is the most dose effective with only 0.4 mg being used and this therefore minimizes side effects, but is the most invasive, and hazardous with potential risks of uterine infection, haemorrhage and rupture of membranes (Calder, 1990). When the outcome of all the routes was compared (Keirse, 1990), the oral and extraamniotic ones were the more unpredictable, leaving the endocervical and vaginal routes giving the most promising results. The endocervical administration in a

viscous gel (injected within the cervical canal) is less invasive and hazardous than the extra-amniotic method and it may allow the PGE<sub>2</sub> to reach the target better and therefore a much smaller dose (0.5 mg) is required than with the vaginal route. However this route is not appropriate for parturition induction when the cervix is already effaced, as the PG placed in the cervical canal may not remain there. Another disadvantage of this route is that PGE<sub>2</sub> is less stable in liquid vehicles than in solid form, and therefore tablets and pessaries may be better than gels (MacKenzie, 1981). The vaginal route is very simple and comfortable to use, but much of the PG may be absorbed systemically, so larger doses are required and side effects have been reported occasionally. Vaginal discomfort is only occasionally reported and usually is not long lasting or severe (MacKenzie, 1981).

In the sheep, the pharmacological ripening of the cervix would be of interest not only to evacuate the uterus, but to allow cervical passage. This is prevented by the presence of four to six "folds" or "rings" that the mucous membrane forms in ruminants (Kristinsson & Wilßdorf, 1985; Rinhold, Rommel and Schulz, 1987; Moré, 1984; Halbert, Dobson, Walton, and Buckrell, 1990). These funnel-shaped folds, with the smallest orifice pointing caudally are concentrated in the caudal part of the cervix. They are out of alignment and the most eccentric ring tends to be the 2nd (Moré, 1984) or the 3rd (Halbert et al., 1990) thus impeding cervical catheterization in this species. As the transcervical route in the ewe is virtually impossible with conventional methods, surgical intrauterine techniques have to be used for AI with frozen semen and ET in this species.

The use of local PGE<sub>2</sub> to help dilate and therefore make it easier to catheterize the cervix despite its high price, could be a possibility for expensive donors if a

consistent effect can be produced during the luteal phase when the flushing is usually carried out. Some attempts to use PGE for this purpose have already been carried out using the vaginal route, both in anoestrus (Rickords & White, 1988) and on day 7 of the pregnancy (Barry, van Niekerk, Rust, and van der Walt, 1990) in sheep and in goats (van Niekerk, Barry, Rust and van der Walt, Langenhoven, 1990). The use of an intravaginal lipid-based suppository containing 10 mg of Dinoprostone (PGE<sub>2</sub>) (Rickords & White, 1988) showed that PGE can induce cervical dilatation, although this was only partial, occurring in most cases at the external os. Complete dilatation and catheterization was possible in only one of twenty ewes. The combination of oestrogens with a double treatment of PGE<sub>2</sub> (Barry et al., 1990) had a better outcome, resulting in successful catheterization (10/10) and high embryo recovery (65%). The drug did not seem to be detrimental to the embryos' viability, as showed by the pregnancy of all 8 of the recipients. The technique consisted in applying to the external os, a solution of 1 mg PGE tablets diluted in 3 ml of saline. At the same time, intramuscular injections of 1 mg of oestradiol cyprionate were given. Twelve hours later (12 h before the embryo collection), after the priming effect of these two drugs, the administration of PGE was repeated, and this time the PGE was applied deeper into the cervix. The ET were carried out under general anaesthesia using cervical manipulation.

PGE<sub>2</sub> in sheep, as in humans, seems to have both pharmacological and physiological actions in the ripening of the cervix during pregnancy and at term (Challis & Olson, 1988). An essential difference between these two species is that in sheep, placental 17 $\alpha$ -hydroxylase metabolizes progesterone or pregnenolone to oestrogens near term, which, in turn seem to stimulate prostaglandin synthesis, whereas the human placenta lacks this enzymatic activity (Liggins,

1983). In late pregnant sheep there are various studies suggesting the involvement of PGs in cervical ripening at parturition. Intraaortic, intravenous or intracervical administration of  $\text{PGF}_{2a}$  and  $\text{PGE}_2$  at term induced cervical softening, although with variable results (Liggins, Fairclough, Grieves, Forster and Knox, 1977; Fitzpatrick, 1977; Fitzpatrick & Liggins, 1980; Ellwood, Anderson, Mitchell, Murphy, and Turnbull, 1981). The cervix by itself, can produce prostanoids ( $\text{PGE}_2$ ,  $\text{PGF}_2$ , Prostacyclin and Tromboxane  $\text{B}_2$ ) both in vivo and in vitro and changes in the pattern of this production occurs at delivery with generalised increase in prostanoids, specially  $\text{PGE}_2$  and Prostacyclin (Ellwood et al., 1981). The role of PGs has also been demonstrated indirectly by studies of the effects of the prostaglandin synthetase inhibitor, its administration at term (Owiny, Fitzpatrick, Spiller and Appleton, 1987) or during labour (Mitchel & Flint, 1978; Ledger, Webster, Anderson and Turnbull, 1985) inhibits cervical softening in sheep. Epostane, an inhibitor of the synthesis of progesterone, when administered alone had proved to induce normal delivery with cervical softening and uterine contractions but the pharmacological inhibition of PG synthesis, prevented the softening effect of epostane. These results indicated that prostaglandins mediated the dilatatory effect of epostane on the cervix (Ledger et al., 1985).  $\text{PGE}_2$  may have a primary role in the regulation of cervical softening in the sheep at term, acting directly on the cervix and not by the induction of uterine contractions. This is supported by Stys, Dresser, Otte and Clark (1981) who demonstrated that the intracervical administration of 30 mg of  $\text{PGE}_2$  to late pregnant sheep was able to increase cervical elasticity, which was monitored by the cervical compliance in vivo. An indirect effect could not be ruled out in that case because 3 out of 8 animals progressed into labour, but other authors (Ledger,

Ellwood and Taylor, 1983; Owiny & Fitzpatrick, 1990) have demonstrated that PGE<sub>2</sub> can soften the cervix in the absence of uterine contractions, and therefore PGE<sub>2</sub> has a direct effect on the cervix. Ledger et al. (1983) induced an increment in the extensibility of the cervix in late pregnant sheep by the infusion of 10 mg PGE into a cervical artery. Owiny & Fitzpatrick (1990), using a smaller dose (3-6 mg) of an intravaginal PGE<sub>2</sub> gel by day 140 of pregnancy (i.e., a week before term) obtained a marked degree of softening (measured in vitro) in the absence of uterine contractions or changes in progesterone concentrations. Suppression of uterine activity using Clenbuterol did not prevent cervical softening at term, indicating that uterine contractions are not essential for cervical ripening (Owiny & Fitzpatrick, 1992). In the same study, the inhibition of PG synthesis prevented cervical softening despite the low levels of progesterone present.

Ultrastructural changes associated with spontaneous cervical ripening during pregnancy and at term in the ewe show similar changes to those observed in humans (Aughey, Munro, Calder, Coutts, and Fleming, 1981; Aughey, Calder, Coutts, Fleming, McManus, and Munro, 1983; Parry and Ellwood, 1981; Fosang, Handley, Santer, Lowther and Thorburn, 1984; Owiny, 1986, Owiny et al., 1987).

A pessary designed for human use was available for the present work. The pessary consisted on a polymer designed to release PGE<sub>2</sub> at a steady rate of less than 1 mg per hour over 8 to 12 hours. Once inserted into the vagina, the pessary swells and rehydrates at a controlled rate, due to the design of the polymer and the shape of the pessary, releasing the active constituent.



The aims of the present study were twofold:

1) The clinical assessment of whether this pessary could facilitate the cervical passage during early pregnancy or the follicular phase in sheep, using different regimes, to study the possibilities of its application in non-surgical AI or ET programs.

2) To investigate whether the cervical ripening induced by this pessary during the luteal phase in the sheep, mimics the physiological cervical ripening of the ewe. Ultrastructural morphology of the cervix in PGE<sub>2</sub>-induced cervical softening, using both Histology and Scanning Electron Microscopy was studied to compare with the pattern described in previous works for the spontaneous softening at term and labour (Aughey et al., 1981; Aughey et al., 1983; Owiny, 1986; Owiny et al. 1987).

## 3.2 MATERIAL AND METHODS

### 3.2.1 Experiment 1

15 superovulated and mated ewes were examined at day 18. Plasma progesterone levels were measured and the sheep pregnancy status determined. The ewes were allocated into 4 main groups. Evaluation of cervical dilatation was carried out by probing the cervix with a 0.5 cm diameter insemination pipette with an angled tip and divisions of 1 cm. The depth scored was recorded before and after the various treatments and the control ewes were examined at corresponding times ( +4.5 and +24 hrs).

1) group C: control group in which no treatment was administered (n= 4).

2) group E: received 0.25 mg of Oestradiol Benzoate, injected IM (n= 3). The effect was evaluated at +20 hours.

3) group P: received either one or two intravaginal pessaries containing 10 mg of Dinoprostone (PGE<sub>2</sub>, (Propess), Roussel Lab) (n=5). The effect of the 1st pessary was evaluated at +4.30 hrs (n=5) and +24 hrs (n=5). A 2nd pessary was inserted into three of the sheep at +24 hrs and evaluated 4 hrs later at +28 hrs (n=3). The two sheep receiving only one pessary were also evaluated at +28 hrs .

4) group PE: this group (n=3) received one pessary of PGE<sub>2</sub> that was evaluated at +4.30 hrs, at this time the ewes were given an intramuscular injection of 0.25 mg oestradiol benzoate. They were evaluated at +24 hr, a second pessary was inserted immediately after this and evaluated at +28 hr (4 hr later) and +48 hr.

### 3.2.2 Experiment 2

Oestrus was synchronized in nine ewes with progestagen sponges containing 60 mg of medroxyprogesterone acetate (MPA) (Veramix, Upjohn Ltd.) for 14 days. At sponge removal the sheep were superovulated routinely with an injection of 1500 I.U. of PMSG. The experiment was carried out in the luteal phase of the cycle (day 6, day 0 being the day of sponge removal and PMSG injection).

Three ewes acted as controls and the remaining 6 received an intravaginal pessary containing 10 mg Dinoprostone. To insert the pessary in the vagina, the hind limbs of the ewes were lifted, the cervix visualized with a vaginoscope and the pessary placed next to the external os with the help of long forceps. The 6 treated ewes were placed into three treatment groups and each given a single pessary for different lengths of time:

1: 8 hours (n=2)

2: 16 hours (n=2),

3: 24 hours (n=2).

Sheep were anaesthetised with 6% Pentobarbitone sodium (Sagatal, RMB) and catheterization was attempted with a catheter of 2 mm diameter to assess the possible dilatation of the cervical canal at the different times of the treatments or their corresponding times in control ewes. They were then sacrificed with 20% Pentobarbitone sodium (Euthetal, RMB). Tissue was taken immediately from three different parts of the cervix: (A) internal, (B) middle and (C) external os; and both (D) anterior and (E) posterior vagina for Histology and Scanning Electron Microscopy (SEM).

### **3.2.3 Microscopy processing**

#### **3.2.3.1 Light Microscopy (LM)**

The tissues were fixed in Bouin's fluid (75 ml of saturated aqueous Picric acid, 25 ml of Formalin and 5 ml of Glacial acetic acid) for 24 hours and processed routinely for paraffin sections of 5 microns. The sections were stained with Haematoxylin/Eosin, Martius Scarlet Blue (M.S.B.), Periodic Acid Schiff (PAS) and Alcian Blue/PAS. The micrographs were taken in a Olympus Vanox-S microscope.

#### **3.2.3.2 Scanning Electron Microscopy (SEM)**

Samples 5 mm wide were taken from the cervix and vagina walls and submerged in containers of modified Karnovsky's fixative (2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M sodium cacodilate buffer) for 24 hours. Specimens were trimmed to expose surfaces to be scanned and washed in buffer (pH 2) for at least 4 hours. The material was dehydrated in ascending concentrations of acetone before critical point drying in carbon dioxide in a drier (Bio-rad machine). The specimens were mounted on aluminium stubs and sputter coated with gold/palladium in a EM Scope SC 500 sputter coater and examined in a Philips 501 B scanning electron microscope.

### **3.3 RESULTS**

#### **3.3.1 Experiment 1**

##### **3.3.1.1 Pessary retention**

In Experiment 1 most pessaries (7/8) were seen to be present in the vagina with a vaginascope 4 hours after placement. After 24 hours, most ewes did not appear to have retained the pessary, although sometimes they were covered by the vaginascope and it was difficult to visualise them because of their transparency.

##### **3.3.1.2 Passage of the Cervix after the treatment with $\text{PGE}_2$ and/or $\text{E}_2$ (Tables 3.1, 3.2, & 3.3)**

The length of the catheter that could be passed into the cervix was variable in the control group, with a range of 0.1 to 3 cm. Variation was observed both between ewes and within the same ewe, when observed at different times.

Some of the treated ewes (in P group) scored 4 cm and 5 cm, which was above the maximal figure observed in the controls (3 cm). With the administration of both  $\text{PGE}_2$  and  $\text{E}_2$  (PE group), none of the ewes scored more than 3 cm. The maximal effect was observed after the administration of a single pessary for 24 hours (group P), with all the ewes having 4 cm. The maximal figure (5cm) was observed in a single ewe(P3) treated with two pessaries. However, in the other two ewes (P4 and P5), the score after 24 hours was greater than found 4 hrs later, after insertion of the second pessaries.

		Cervical depth(cm)		
ewe #	pregnant	+0.00 hr	+4.30 hr	+24.00 hr
C1	+ve	3.0	0.1	-
C2	+ve	3.0	3.0	1.0
C3	+ve	0.1	2.5	1.0
C4	-ve	0.1	2.5	1.0

Table 3.1 Cervical depth measured at time intervals following mock pessary insertion in control group.

		Cervical depth (cm)			
ewe #	pregnant	-28.30 hr	-24.00 hr	+0.00 hr	+20.00 hr
E1	+ve	-	3.0	-	0.5
E2	+ve	3.0	3.0	1.0	2.5
E3	+ve	0.1	3.0	1.5	4.0

Table 3.2 Cervical depth measured at time intervals following oestrogen treatment (Group E)

ewe #	pregnant	Cervical depth (cm)			2nd pessary inserted [+24.00 hr]	Cervical depth (cm)	
		+0.00 hr	+4.30 hr	+24.00 hr		+28.00 hr	+48.00 hr
P1	+ve	0.1	0.1	4.0	no	3.0	-
P2	+ve	-	4.0	4.0	no-	3.0	-
P3	-ve	0.1	1.5	4.0	yes	5.0	-
P4	+ve	0.1	0.5	4.0	yes	1.5	-
P5	+ve	0.1	3.0	4.0	yes	2.0	-
PE1	+ve	-	3.0	2.0	yes	1.0	-
PE2	-ve	3.0	*2.0 <sup>1</sup>	2.0	yes	3.0	2.0
PE3	+ve	-	*3.0	2.0	yes	2.0	2.0 <sup>1</sup>

\* 0.5mg oestradiol benzoate given at +4.30 hr

<sup>1</sup> pessary found to be missing

Table 3.3 Cervical depth in group P (PGE pessary) and group PE (PGE pessary and oestradiol) at time intervals following treatment.

### **3.3.2. Experiment 2**

In Experiment 2 when the tract was dissected, in three out of six ewes the pessary was absent at the time of the assessment of the PGE<sub>2</sub> effect (8 and 24 hours). In another ewe (after 16 hours) this was not recorded and therefore in only two ewes (#8 and #7) was the pessary known to have been releasing PGs for 8 and 16 hours respectively.

#### **Physical Changes after PGE<sub>2</sub> compared with controls. (Table 3:4)**

The cervixes felt very soft in the treated sheep when the specimens were cut. Only in ewe #6 which had had a pessary for 8 hrs or less was the cervix harder than in the rest. In the control group, one had a very hard cervix (#2). The other two controls had softer cervixes but were still harder than the treated group.

#### **3.3.2.1 Ultrastructural studies**

##### **a) Histology of the Cervix**

##### **a1) Changes in the Connective Tissue**

##### **Treated versus Controls**

The difference in the connective tissue of the cervix between both treated and control groups is illustrated with micrographs of representative specimens (controls 4a,b,c versus treated 5a,b,c). In the control sheep, dense connective tissue was observed characterized by the predominance of densely arranged collagen fibres over the other components such as ground substance and cells. The fusiform fibrocytes with dense pycnotic nuclei predominated over the fibroblasts, an active secretory cell characterized by a bigger oval nucleus. In the treated sheep this tissue changed from being highly fibrous to having a more cellular nature.



ewe #	time of treatment [hrs]	pessary retained	cervical consistency*	cervical catheterization success
5	<24	no	3	no
4	<24	no	3	no
6	<8	no	2	no
7	16	yes	3	NA
9	16?	NR	NR	NA
8	8	yes	3	NA
2	-	-	0	NA
3	-	-	1	NA
1	-	-	1	NA

\* Softening of cervix. A subjective assessment relating the consistency of the cervix at cutting to that in ewe #5. (The lower the number, the harder the cervix)

NR = Not recorded

NA = Not attempted

Table 3.4: Experiment 2: Physical changes to cervix following PGE2 pessary treatment

histological changes in the CT of cervix													
ewe #	pessary found	PGE treatment hr	collagen			fibroblasts			fibrocytes			ext os	
			int os	mid	ext os	int os	mid	ext os	int os	mid	ext os		
5	no	<24	2	2	2	4	4	-	1	1	-		
4	no	<24	4	4	>3	-	2	-	-	3	-		
7	yes	16	4	4	>3	3	3	4	3	2	1		
9	?	<16	5	4	3	3	1	3	2	4	2		
8	yes	8	4	4	3	2	2	4	3	3	1		
6	no	<8	5	4	4	4	3	3	4	4	3		
2	-	-	5	5	5	1	1	1	5	5	5		
3	-	-	5	5	5	1	1	1	5	5	5		
1	-	-	5	5	5	1	1	1	5	5	5		

Scores 1-5 (5: maximal occurrence and 1: minimal). Differences between treated and controls and between the portions of the cervix in treated ewes.

Table 3:5. Occurrence of the components of cervical connective tissue.

ewe #	treated(T) or control(C)	length of pessary treatment (hr)	internal cervical os*	mid- cervix*	external os*
1	C	0	1	2	3
2	C	0	1	1	3
3	C	0	1	1	3
4	T	24	2	2	3
5	T	24	2	3	3
6	T	<8	0	2	2
7	T	16	3	3	3
8	T	8	3	3	3
9	T	<16	3	3	3

\* 0 = no stratification

\* 1 = stratified columnar epithelium in crypts

\* 2 = present in some cervical crypts (stratified squamous)

\* 3 = extensive areas covering both crypts and villi (stratified squamous)

Table 3.6 The scores of the degree of stratification of the epithelium of various parts of the cervix, following intravaginal treatment with PGE2.

Collagen was looser, the collagen bundles separated between them and into fibres, which gave the impression of an apparent increase in the proportion of ground substance. The cellular component was more prominent in this group of ewes and the ratio of fibroblast: fibrocytes shifted into a predominance of fibroblasts.

The changes above described in the treated ewes were not constant throughout the length of the cervix, more marked changes being found next to the vagina (at the external os) than in the deeper portions of the cervix, (mid cervix and internal os respectively) (Plate 4 a,b and c). In control ewes the pattern of the connective tissue did not differ between the three portions of the cervix studied (Plate 5, a,b and c). Scores of the occurrence of the different components of the connective tissue were given on a scale of 1-5 (Table 3:5). Treated ewes differed more from the controls at the external os than at the internal os, mid cervix having intermediate scores.

#### **Differences between the duration of the PGE<sub>2</sub> treatment.**

A gradation in the changes already described was established among the individual ewes, according to three criteria:

- 1) The score in physical changes already described (Table 3.4).
- 2) The score in Microscopical changes of the Connective Tissue (Table 3.5). High scores in the occurrence of Fibroblasts and a low score in the occurrence of Collagen fibres and Fibrocytes were indicative of more changes in comparison to Control ewes.
- 3) The depth of changes within the cervix.

Changes found at the internal os were considered indicative of a greater action of PGE<sub>2</sub>.

A certain pattern relating the magnitude of the changes within the Connective Tissue with the duration of the administration of  $\text{PGE}_2$  could not be precisely established because some ewes lost their pessaries. However, the CT changes were maximal at the internal os in ewes planned to be treated for 24 hour compared with the ones planned for 8 hours. The sequence organized according to an increasing  $\text{PGE}_2$  effect was as follows: #6 (<8 hrs), #9 (<16 hrs), #8 (8 hrs), #7 (16 hrs), #4 (<24 hrs) and finally the maximum action in #5 (<24 hrs).

## **a2) Subepithelial infiltration in the Cervix**

Infiltration of the subepithelial tissue with white blood cells (WBC) such as Lymphocytes, Plasma cells and Polymorphonuclears, was not exclusive of any of the groups. WBC were present in both control (fig 1b) and treated sheep groups (micrograph not shown).

## **a3) Changes observed in the Epithelium of the Cervix (Table 3.6)**

Within the control group, the simple columnar epithelium merges abruptly with stratified squamous at the external os, with overlapping of both epithelia (micrographs 3a and 3b). A stratified epithelium was observed at mid cervix occasionally (fig 2b), but only in the crypts (micrographs 2b) and not at the internal os of the cervix in this group. The histological sections revealed that the structure of the epithelium changed within the treated animals, from the typical high columnar into another more similar to the stratified squamous epithelium of the vagina. The number of layers increased, the tall columnar cells being substituted by big polyhedral cells that were undergoing a process of considerable desquamation. These changes reached the internal os of the cervix in three cases (#7, #8 and #9; micrographs 1c and 1d).

The presence of epithelial mucins demonstrated with PAS, resulted in the normal deep purple red, mucins were located within the goblet cells in areas of normal epithelium (2b), arranged in one layer of drops. In the areas of abnormal epithelium, the abnormal big round cells were surrounded by mucins at the top of the epithelium (1c and 1d). The character of mucins secreted by the goblet cells did not differ between groups, it was a mixture of acid and neutral that stained in a bluish deep purple colour with the Alcian Blue/PAS stain.

A gradation of changes in the epithelium seemed to exist and parallel the CT changes. The normal tall columnar epithelium with goblet cells was seen only in the ewe #6 (<8 hrs), which had few changes of CT. In the rest of the sheep, scarce areas of normal epithelium were seen.

#### **b) Histology of the Vagina.**

The epithelium in both treated and control was the typical squamous stratified, the superficial squames of this epithelium were rounded (no micrograph shown), similar to the squamous epithelium of the external os.

The changes in the CT in treated ewes were similar to the ones described for the cervix (fig 5d).

#### **c) SEM of the Cervix**

##### **c1) Epithelium**

##### **Control group**

The internal os was lined with Columnar Epithelium exclusively. Transitory epithelium was found in one of the control ewes (#1) at mid cervix, but most of the

epithelium was columnar in this area. Only the apex of the densely packed cells can be seen with SEM. In most areas, two types of columnar cells were differentiated in the epithelial surface of the internal os and mid cervix: 1) ciliated cells and 2) secretory cells, characterized by convex apex and the presence of microvilli on their membranes (micrographs 6a and 8a). Occasionally, some areas were dominated by the presence of dilated secretory cells of spherical shape (micrograph 6b). These cells were in their developmental stages, with stretched membranes.

The presence of mucus within the cervical canal differed between folds. The more extensive areas of cervical folds had no mucus outside the secretory cells, which allowed the differentiation of the two types of cells of the epithelium (6a and 8a). Other small areas had only a thin strand of mucus overlying the epithelium (8a). In other areas, the tips of the cells were completely covered by a thin layer of mucus (8b) and an undulating surface due to the folds and dome-like shape of the columnar cells was still obvious. Finally, the mucus was so thick in some other areas, that the shape of the epithelium was totally masked and appeared flat.

The observation of the epithelial surface of the Cervix by SEM, revealed two types of squamocolumnar junction at the external os, i.e. patches of both epithelia and a clear border line. At this site some cells that were intermediate in size (10-15  $\mu\text{m}$ ) between the squames (30  $\mu\text{m}$ ) and the columnar cells were observed (4  $\mu\text{m}$ ) (micrograph 7a).

## **Treated animals with PGE<sub>2</sub>**

The typical columnar epithelium of the luteal phase, with dilated cells characterized by the presence of microvilli in most areas that has been described for the control groups, was exceptionally seen in this group at internal os and mid cervix. The columnar epithelium was covered by mucus in most areas in this group. This was especially copious in sheep #7 (16h) and #6 (<8 h) in the external and mid cervixes. Different stages of its secretion were seen in the various folds: small particles of mucus (8c), fibres, a reticular form (8c) and extensive layers of mucus (8d).

Some additional features were unique of this group such as:

- 1) Desquamation of the columnar epithelium, observed in some ewes (#5, #4, #9, #8). SEM also showed columnar cell separation and desquamation (7b and 9c).
- 2) Presence of round, big non-epithelial cells that may be WBC (Micrographs 9a, 9b and 9c). Some RBC were seen in sheep Nos #4 (<24h) and #6 (<8h).
- 3) Presence of isolated squamous cells at the int os and mid cervix (9a and 9b).

## **c2) Connective Tissue**

### **Treated animals with PGE<sub>2</sub>**

In all the treated ewes, different degrees of separation of the big collagen bundles were observed (11b and 11d) when compared with the control group in which only slight separation of the individual fibres was observed and no big bundles could be differentiated (Picture 11a and 11c). The separation was more accentuated at the external os of the cervix in most ewes.



#### **d) SEM of the Vagina**

##### **d1) Epithelium**

The vagina in Control ewes was lined by the typical squamous epithelium (micrograph 10b) although some small areas of columnar secretory epithelium were observed in the cranial portion (10a). The surface of the vagina was smooth, covered by large, flat, polygonal cells with central nuclei and raised boundaries between the adjacent cells (micrograph 10b). Anastomosing microridges were obvious in most cases and part of the epithelium was covered by mucus. In some cases other non-epithelial cells of round shape and 3  $\mu\text{m}$  in diameter were observed which were probably red blood cells.

In the treated animals with  $\text{PGE}_2$  a more intense desquamation was observed in the squamous epithelium, specially in the cranial portion of the vagina. In some cases there were round holes in the epithelium. Round shaped cells, non-epithelial were also commonly observed in the surface of the epithelium, usually associated with abundant mucus. A secretory epithelium with cells similar to the dilated cells of the columnar epithelium, was observed in the cranial vagina of various ewes. Cells similar to the dilated secretory cells were observed in one case in the caudal vagina (#5 in micrograph 10d).

**Plate 1 Epithelium of the internal os of the cervix. Control compared with PGE<sub>2</sub>-treated ewes (all at day 6 of a superovulated cycle)**

**Fig 1a) Control ewe (#2). A cervical crypt. (MSB x 20)**

Typical simple high columnar epithelium (Cole), characterized by a single layer of elongated cells with parabasal nuclei which is perpendicular to the basal membrane (BM). Some small areas of the crypts show stratification, with two to six layers of epithelium. The superficial layer is composed of columnar cells and the cells beneath are polyhedral.

**Fig 1c) Ewe treated with PGE<sub>2</sub> for 8 hours (#8). Villi and crypts. (PAS x 20).**

Complete metaplasia with total replacement of columnar cells (as observed in control ewes) by stratified squamous epithelium (SqE) typical of the vagina. Note that the junction between this type of epithelium and the connective tissue at the basal membrane (BM) is irregular rather than perpendicular to the epithelial plane, forming stromal papillae. Note also the augmented thickness of the epithelium and the flat cells (squames) rather than the columnar appearance of the superficial layer. Mucus and red blood cells (RBC) are covering portions of the epithelium

**Fig 1b) Control ewe (#1). Villi of the epithelium. (MSB x 20)**

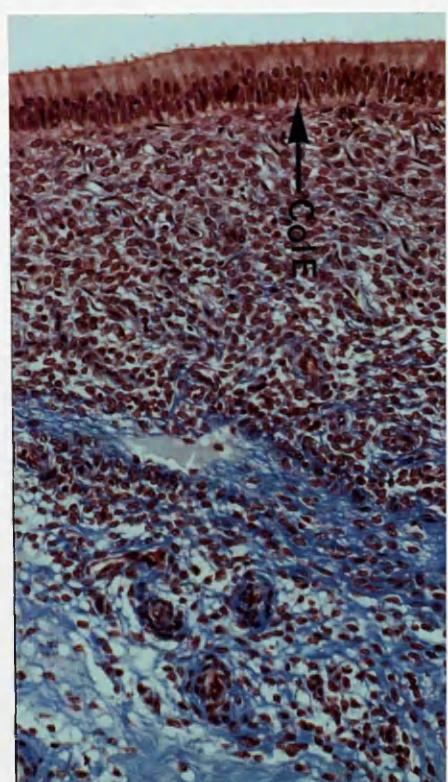
Stratified high columnar epithelium (Cole) with no more than 4 rows of parallel nuclei. The epithelium is constructed of tall, narrow, columnar cells at the superficial row. The columnar cell nuclei are oval to rod-shaped and lie at the base of the cells. Above the nucleus is the secretory portion of the cell or the cilia. Note the subepithelial infiltration with stromal cells. This was present in two of the three control ewes.

**Fig 1d) Ewe treated with PGE<sub>2</sub> for <16 hours (#9). Villi. (PAS x 40)**

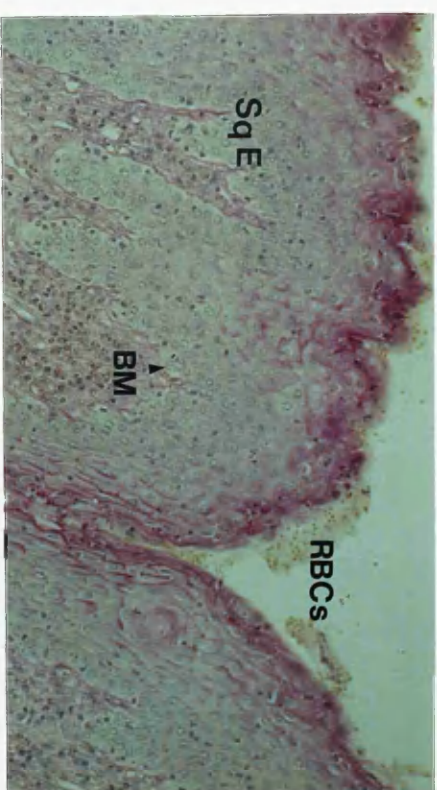
Incomplete metaplasia, which allows comparison of the metaplastic stratified squamous epithelium (SqE) with the normal simple high columnar epithelium (Cole) at higher magnification.



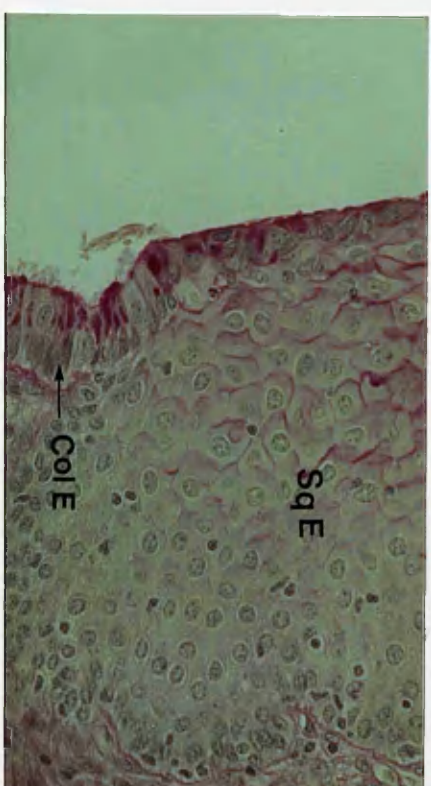
1a



1b



1c



1d

**PLATE 2: Epithelium of mid cervix. Control compared with PGE<sub>2</sub>-treated ewes**

(all at day 6 of a superovulated cycle).

**Fig 2a) Control ewe (#1). Cervical crypts. (AB/PAS x 40).**

Tall columnar epithelium is dominant in this portion of the cervix in control ewes. Mucus (Mu) within the goblet cells and in the cervical lumen is stained a mixture of blue (Alcian Blue) and magenta (PAS positive), indicating that there is a mixture of neutral and acidic mucins. Note the difference in the basal membrane (BM) which is stained only with PAS (magenta).

**Fig 2c) Ewe treated with PGE<sub>2</sub> for 8 hours (#8). Cervical crypt. (AB/PAS x 20).**

Presence of incomplete metaplasia with the columnar epithelium transforming into stratified squamous epithelium. This finding did not differ from the control animals in this portion of the cervix. Mucus is slightly acidic (blue-magenta) when compared with the PAS alone in figure 2d (magenta), but no more acidic than in control ewes (2a). The glycosaminoglycans (GAG) of ground substance are also stained with this technique. Note the different colour compared with controls (2a) indicating changes in the composition.

**Fig 2b) Control ewe (#1). Crypts and villi of the epithelium. (PAS x 20).**

Note the presence of stratified squamous epithelium (SqE), restricted to the crypts at this part of the cervix. Note that the border with the connective tissue (CT) remains parallel to the epithelium and stromal papillae are absent (arrow).

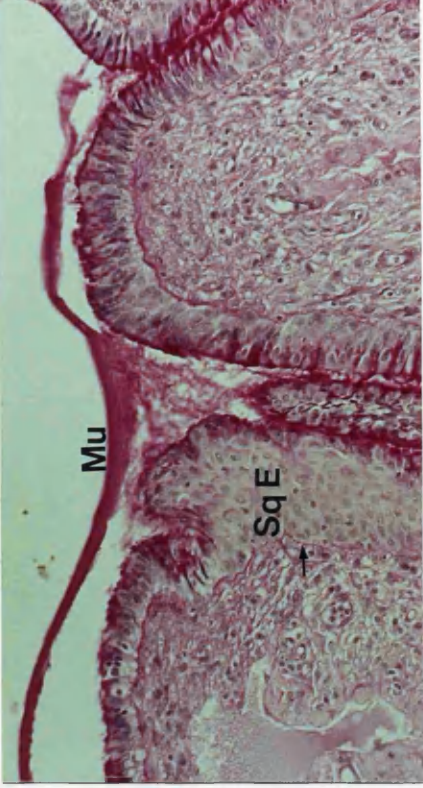
**Fig 2d) Ewe treated with PGE<sub>2</sub> for 16 hours (#7). Crypt. (PAS x 40).**

The stratified squamous epithelium was very common in this part of the cervix, in this case lining a whole crypt. Note that some of the goblet cells (GC), full of mucus, seem to remain in the superficial stratum and in other areas they have been completely substituted by squames (Sq).

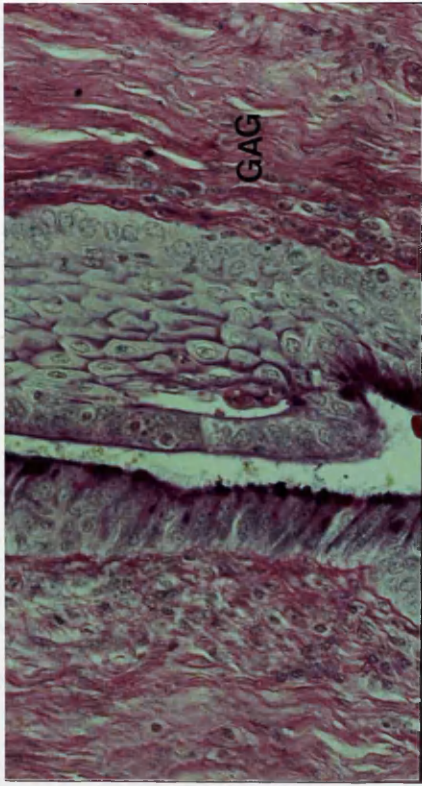




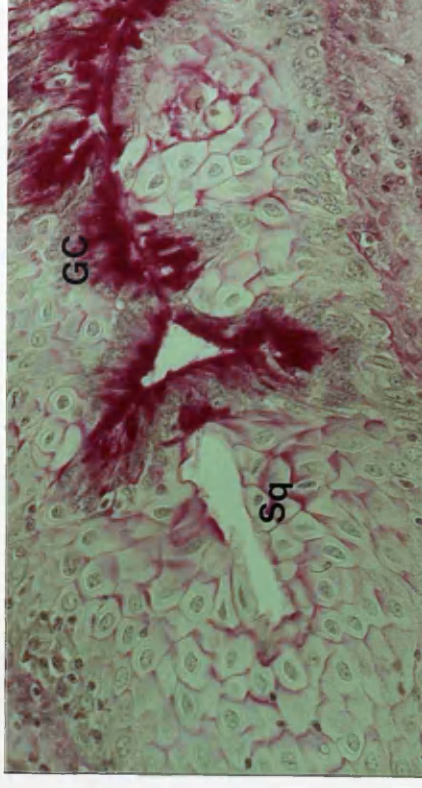
2a



2b



2c



2d

**PLATE 3: Epithelium of the external of the cervix. Control compared with PGE<sub>2</sub>-treated ewes (both groups at day 6 of a superovulated cycle).**

**Fig 3a) Control ewe (#3). Cervical crypt. (MSB x20).**

Transitional epithelium between columnar and squamous with overlapping of both epithelia. Note the inactivity within the squamous epithelium.

**Fig 3b) Control ewe (#2. Crypts and villi of the epithelium. (MSB x 10)**

Note the transitional epithelium in crypts and villi.

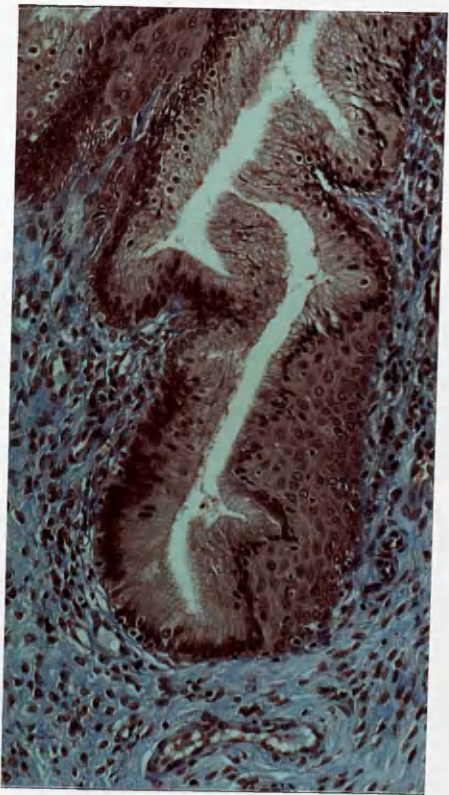
**Fig 3c) Ewe treated with PGE<sub>2</sub> for <24 hours (#5).**

Cervical crypts and villi. (MSB x 10)  
Section shows reactive tissue. Note the presence of numerous vacuoles in the upper strata of the squamous epithelium. Note the double thickness (more layers of cells) of the epithelium when compared with micrograph 3b.

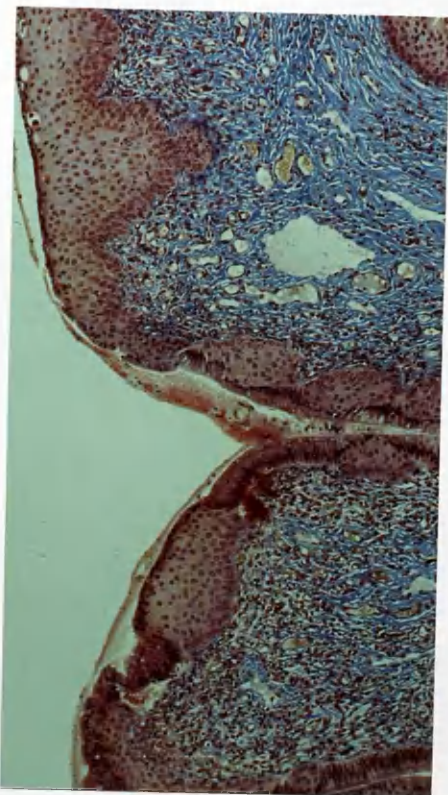
**Fig 3d) PGE<sub>2</sub>-treated ewe (<24 hours) (#4). Crypt. (MSB x 40).**

Same features as observed in Figure 3c, at higher magnification.

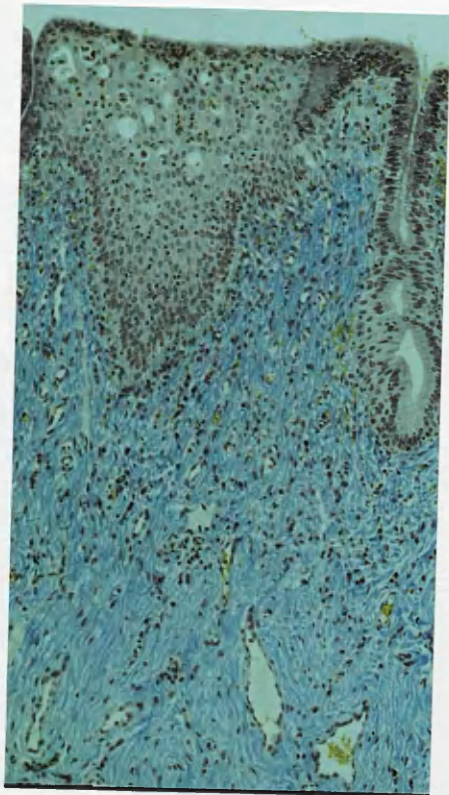




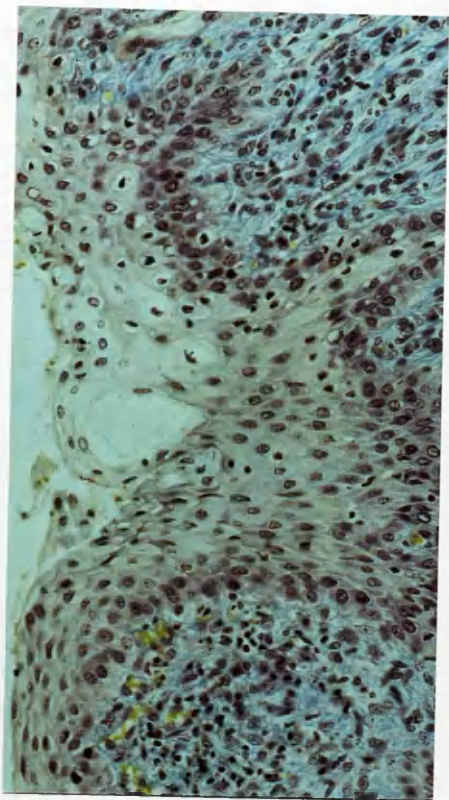
3a



3b



3c



3d

**PLATE 4 Connective tissue in the cervix and vagina of control ewes (MSB x100)**

**Fig 4a) Internal os**

**Fig 4b) Mid cervix**

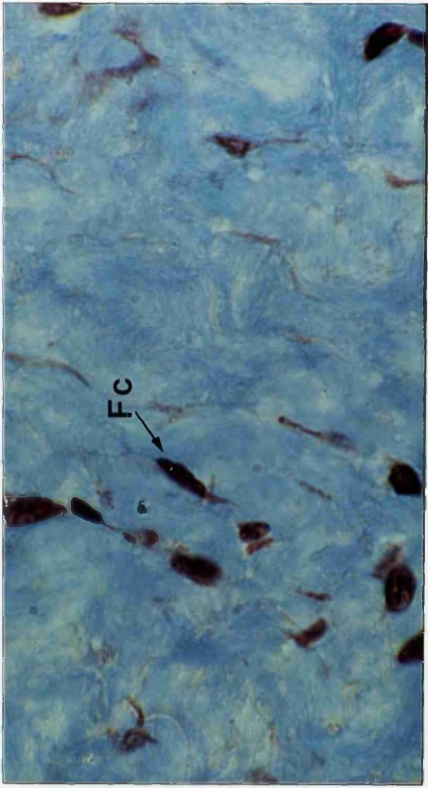
**Fig 4c) External os.**

Note that there are slight differences between the portions of the cervix. They all present a dense connective tissue with predominance of collagen (stained in blue). The cells are sparse and there are more fibrocytes (elongated) than fibroblasts. Note the condensed and elongated nuclei (Fc) of the fibrocytes.

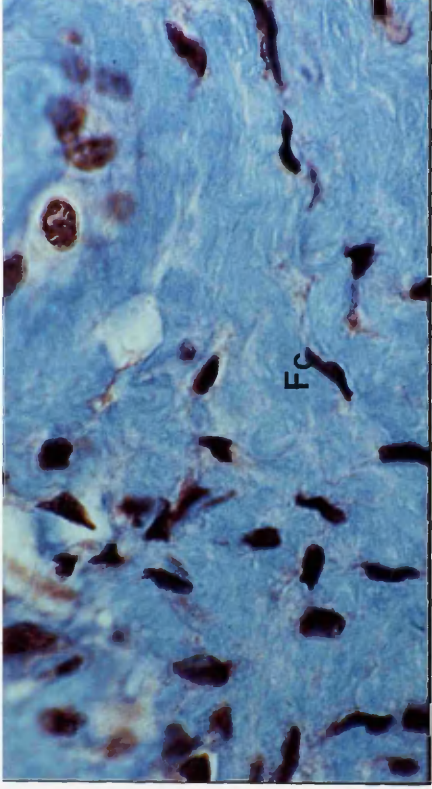
**Fig 4d) Cranial vagina.**

Note that the collagen is slightly less densely arranged in the vagina than in the cervix. Small number of cells, both WBC and fibroblasts. Note the presence of a plasma cell (PC).

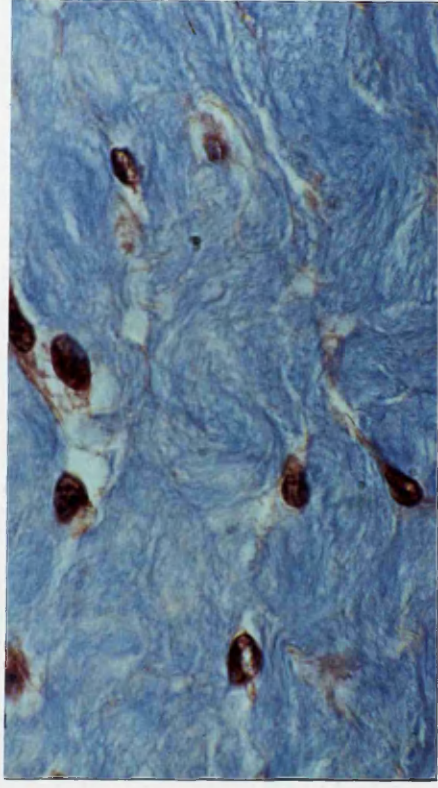




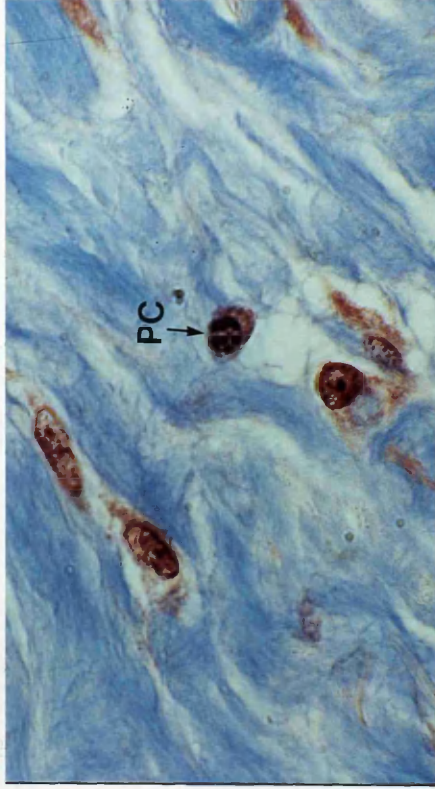
4a



4b



4c



4d

**PLATE 5: Connective tissue in the cervix and vagina of treated ewes. (MSB x100)**

**Fig 5a) Internal os.**

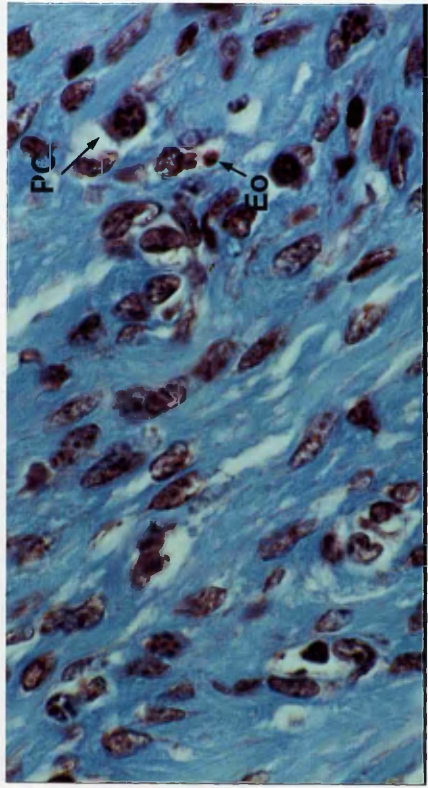
**Fig 5b) Mid cervix.**

**Fig 5c) External os.**

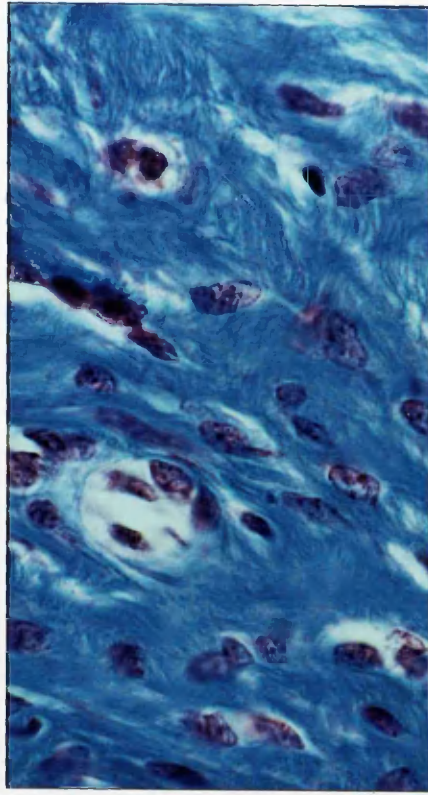
**Fig 5d) Cranial vagina.**

Note the presence of more cells in these photomicrographs than in Plate 4 (controls), especially fibroblasts, which are characterized by their large and rounded nuclei (Fb) and prominent nucleoli (n). Also seen are WBC including plasma cells (PC), lymphocytes (Ly) and eosinophils (Eo) nuclei. There are open spaces between collagen fibres, filled with ground substance. The ground substance is not stained because it dissolves during tissue processing and therefore appears white. Note that the changes of collagen dissociation and the increase in ground substance are gradually more marked caudally, from the internal os (a) towards the vagina (d).

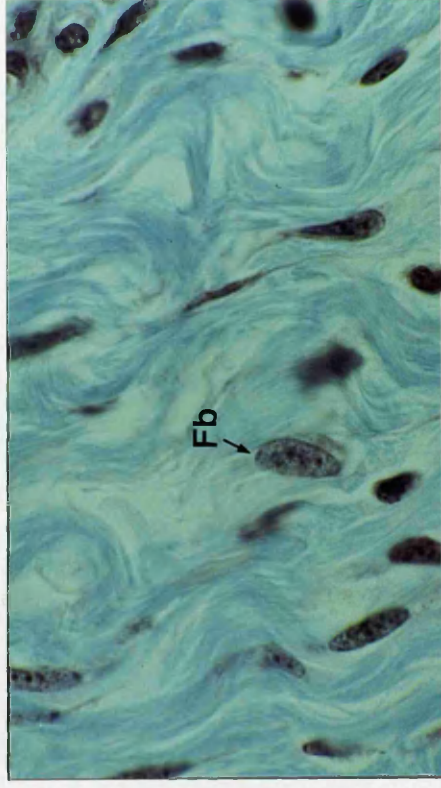




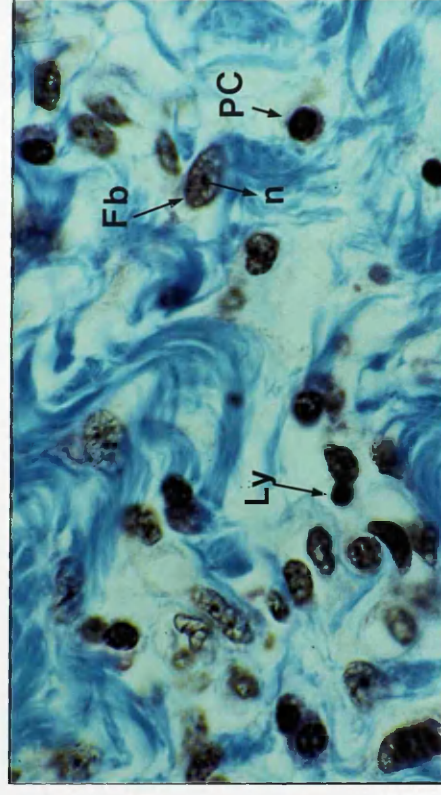
5a



5b



5c



5d

**Plate 6: Columnar epithelium of the cervix in controls compared with treated ewes**  
(day 6 of a superovulated induced cycle)

**Fig 6a) Control ewe (#2) at the internal os (x2500).**

This micrograph shows the typical aspect of the apical view of the columnar epithelium in the luteal phase when 2 types of cells can be differentiated, namely the secretory cells (SC) and the ciliated cells (CC). Note the presence of microvilli (Mi), characteristic of the secretory cells at this stage of the cycle.

**Fig 6b) Control ewe (#1) at mid cervix (x1250).**

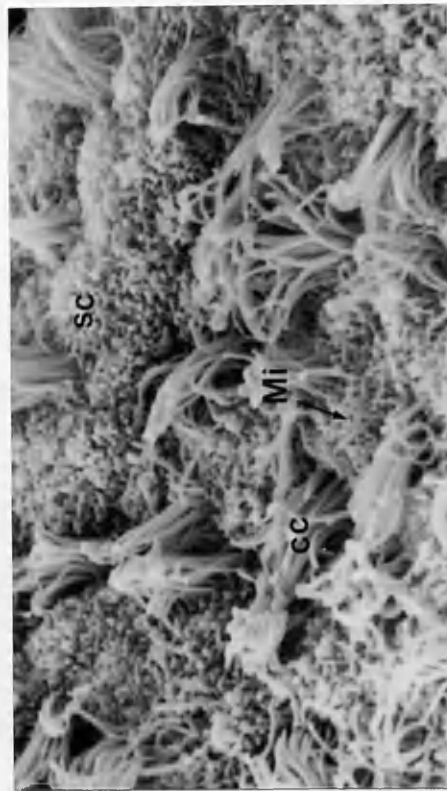
In some areas, dilated secretory cells (DSC) in their developmental stage were observed in this group. The tips of the cells are dilated and exceed the height of the ciliated cells. Some blebs (Bl) released from the secretory cells can be seen over the cilia. Note the variable size of their diameter (1-2 $\mu$ m) which corresponds to various stages of their formation.

**Fig 6c) Ewe treated for 8 hrs (#8). Mid cervix (x2500).**

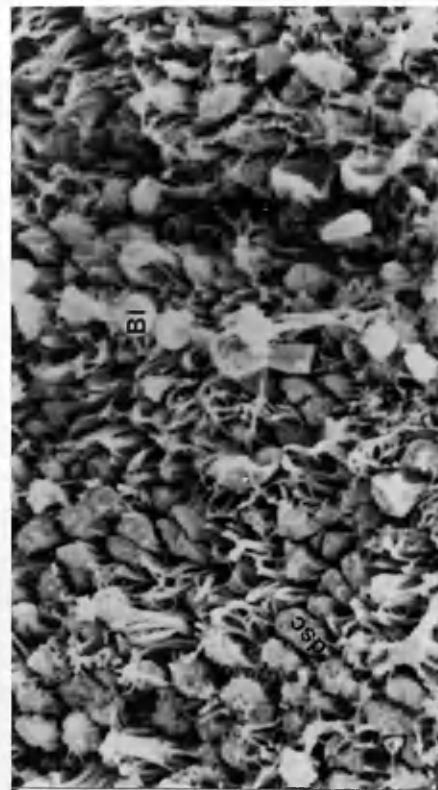
Note the presence of pleomorphic structures (1-4 $\mu$ m), which may be RBC or platelets, over the usual ciliated cells and secretory cells with microvilli.

**Fig 6d) Ewe treated for 24 hr(#4) at the internal os (x1250).**

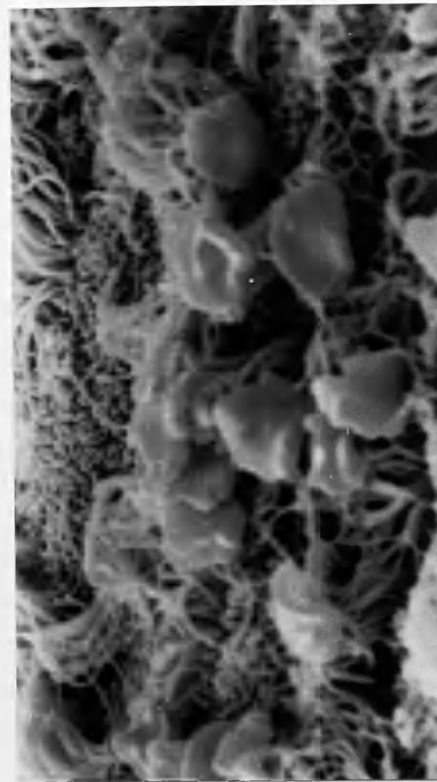
Note the different aspect of this epithelium when compared with controls at the same magnification (6b). Ciliated and secretory cells are not visible. In this "desert-looking" micrograph, the top of these cells is covered by mucus. Pleomorphic structures of variable size (1.2-4  $\mu$ m), similar to the previous micrograph, can be seen here trapped in the mucus.



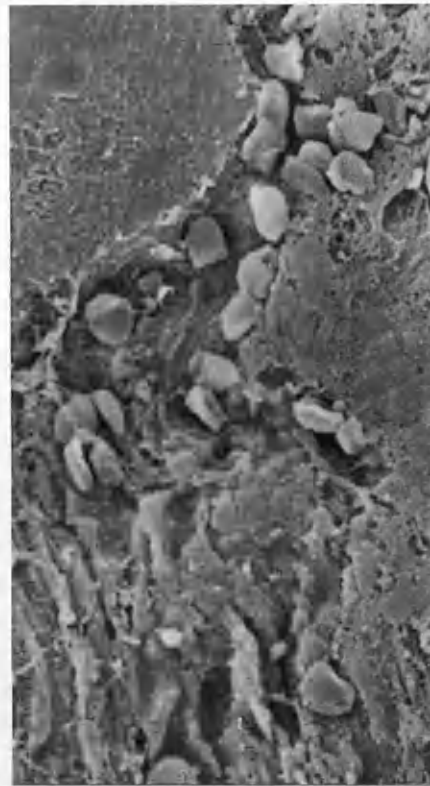
6a



6b



6c



6d

**PLATE 7: Columnar epithelium of the cervix at the external os in controls compared with treated ewes (day 6 of a superovulated induced cycle).**

**Fig 7a) Control ewe (#2). External os (x2500).**

In this zone of the cervix, the ciliated cells were scarce. This micrograph shows only secretory cells, characterized by their dome shape. These cells are very variable both in size (4-15  $\mu\text{m}$ ) and in the appearance of their membranes in this transitional zone. Some of the cells have microvilli and others have small pits and are covered with small particles. None of the cells possessed cilia. It was common to find isolated erythrocytes (RBC), this one measuring approximately 3  $\mu\text{m}$  (Barnhart, 1983).

**Fig 7b) Ewe treated for <8hr (#6) (x1250).**

Secretory cells. Most cells have a size of 14  $\mu\text{m}$ . Note the junctional complexes between secretory cells. Some of the cells are losing cohesion with the rest.



7a



7b



**PLATE 8: Mucus observed on the cervix on day 6 of a superovulated induced cycle in controls and treated ewes.**

**Fig 8a) Control (#1) (x640)**

Typical image of this stage of the cycle (metoestrus), when the mucus (Mu) dissociates and coagulates into strands, uncovering the epithelium and allowing the apices of the columnar cells to be observed, as in plate 6a.

**Fig 8b) Control (#2) (x2500)**

Some areas are still completely covered by a layer of mucus.

**Fig 8c) treated with PGE<sub>2</sub> for <8hr (#6) (x1250)**

Note that in this group of ewes the mucus was forming rather than dissociating. Fibres of mucus are forming from the convergence of the particles secreted by the dilated secretory cells. This picture is often observed at pro-oestrus during the normal cycle.

**Fig 8d) treated for <24hr (#4) (x2500)**

The fibres also coalesce to form an amorphous matrix which covers the epithelium (Mu). This type of mucus is characteristic of oestrus and therefore is atypical of the present stage of the cycle.

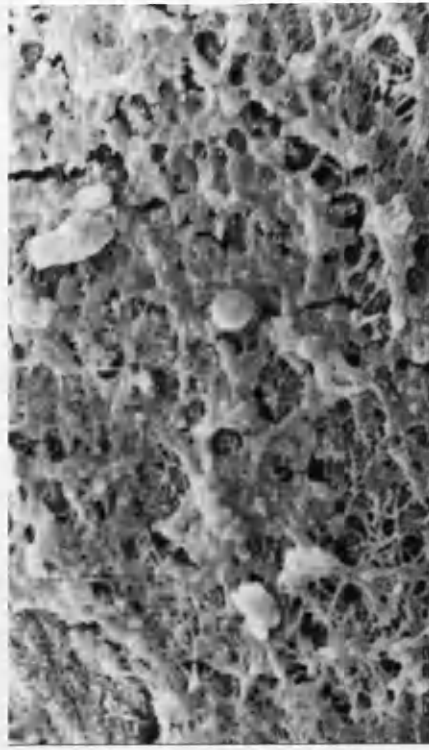




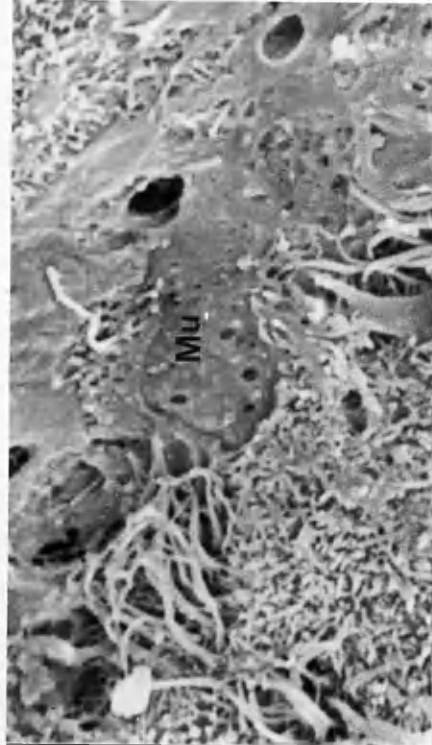
8a



8b



8c



8d

**PLATE 9: Unusual features observed in the epithelium of cervix in treated ewes.**

**Fig 9a) Internal os, treated ewe #8 (8hr) (x640)**

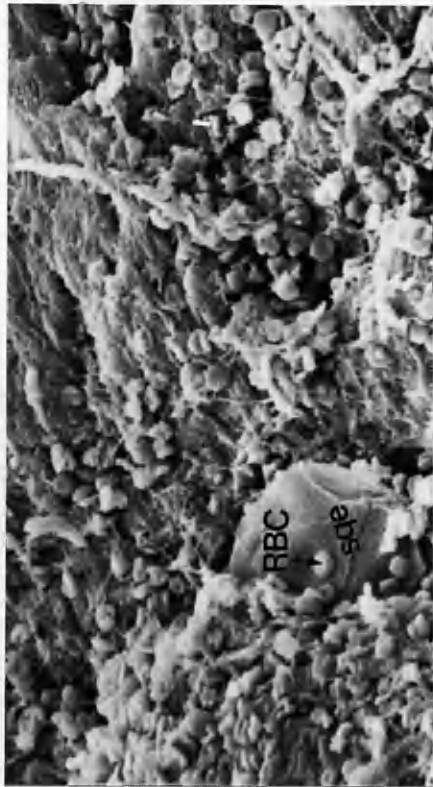
Most of the epithelium is covered by a thick layer of mucus and numerous erythrocytes (RBC). Note the characteristic shape of biconcave discs and the size of their diameter ( $3\text{ }\mu\text{m}$ ). A piece of squamous epithelium (sqe) can be observed at the internal os, which is an unusual site for this type of epithelium.

**Fig 9b) Mid cervix, treated ewe #8 (8hr) (x 1250)**

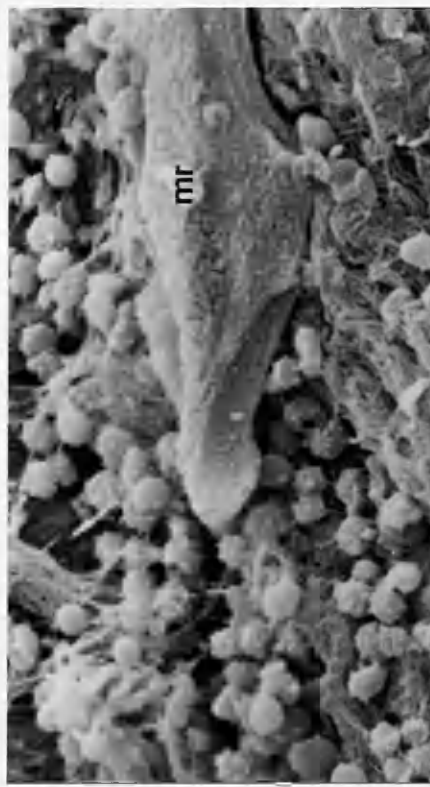
Note the presence of a squamous cell, which can be identified by its characteristic microridges (mr), its voluminous size (more than  $44\text{ }\mu\text{m}$ ) and polyhedral shape. The finding of a squame at mid cervix with SEM is in line with the histological findings (2c and 2d). The round structures ( $5\text{-}6\text{ }\mu\text{m}$ ) may be white blood cells (WBC).

**Fig 9c) External os, treated ewe #8 (8hr) (x 640)**

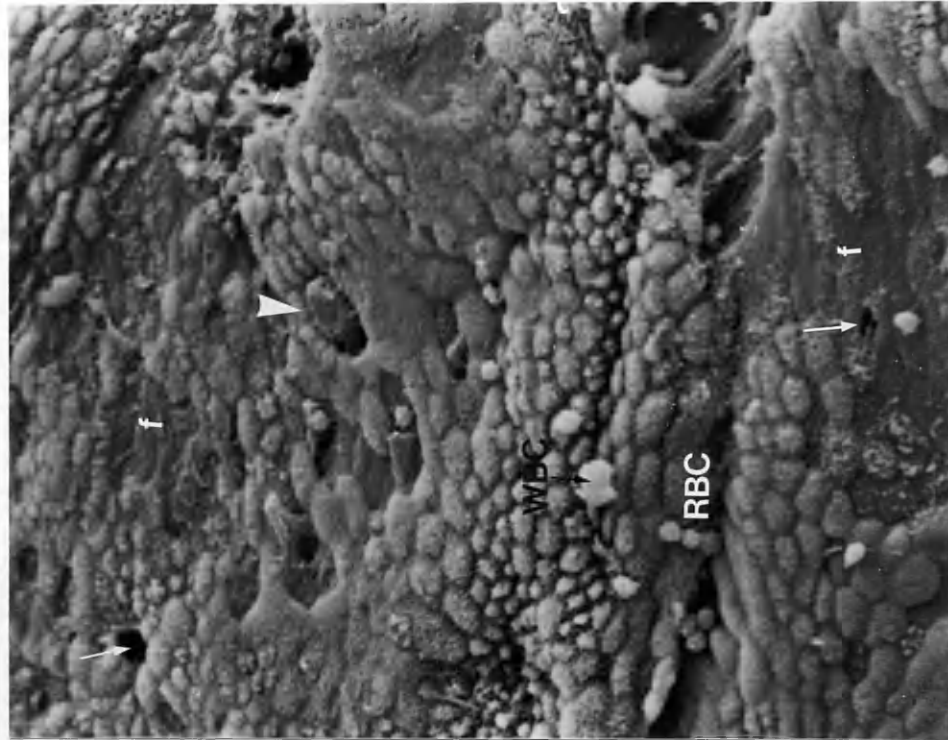
The heterogeneous size of the non ciliated cells was characteristic of this area of the cervix in the present study, as was observed in both groups. Note that in some areas the domes do not protrude and the epithelium is much flatter (f). Note that a columnar cell is losing cohesion (white arrow head) and also the presence of numerous holes (arrow) or gaps between the epithelial cells, which were not observed in control ewes (7a). Note the presence of red (RBC) and white blood cells (WBC) emerging from those gaps.



9a



9b



9c

**PLATE 10: Epithelium of the vagina of controls and treated ewes.**

**Fig 10a) Control ewe (#1), cranial vagina (x2500)**

This micrographs illustrate the presence of secretory cells in cranial vagina. Note, the particles of mucus (Mu) in the plasma membrane and the characteristic junctions between cells. This transitional epithelium was observed in limited areas of the control group. The epithelium is similar to the one found at the external os (Plate 7a), with cells of variable size (4-7  $\mu\text{m}$ ).

**Fig 10b) Control ewe (#2), caudal vagina (x640)**

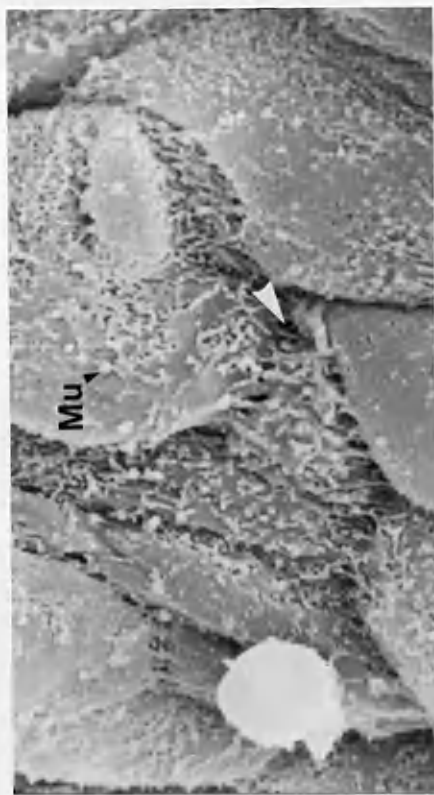
Typical aspect of the squamous epithelium characterized by its smoothness. The squamous cells (sqc) are flat, voluminous (37x14  $\mu\text{m}$ ), polygonal shaped with central nuclei (N) and raised boundaries between the adjacent cells (arrow heads). The small cells with biconcave or spherical shape and 3.7  $\mu\text{m}$  in diameter are RBC.

**Fig 10c) Treated ewe #9 (<16 hrs). Cranial vagina (x1250).**

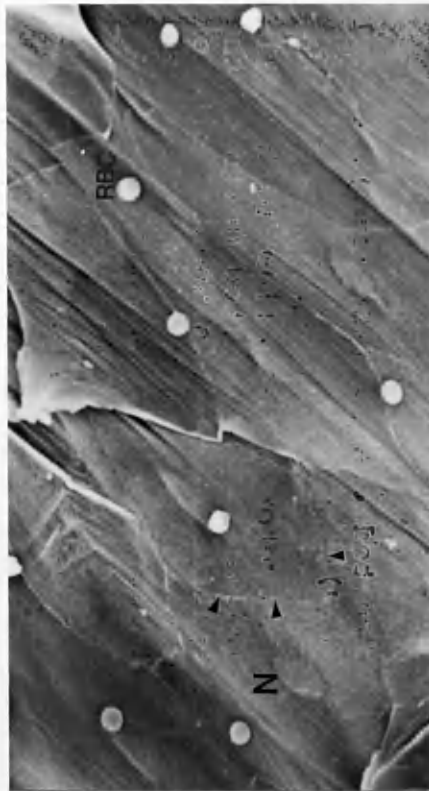
Note the disorganized appearance of this active epithelium in contrast with the smoothness observed in the vagina of controls (10b). Note the intense desquamation, round holes in the epithelium, non epithelial cells of round shape (3 $\mu\text{m}$ ) associated with abundant mucus

**Fig 10d) Treated ewe #5 (<24 hrs.). Caudal vagina (x1250)**

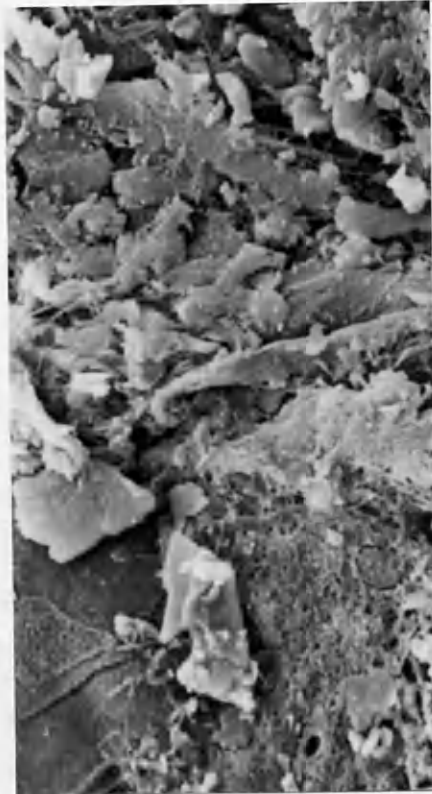
Note the secretory epithelium with cells (10  $\mu\text{m}$ ) similar to the dilated cells of the columnar epithelium in this unusual site of the vagina. Mucus particles (Mu) and junctional complexes (arrow head).



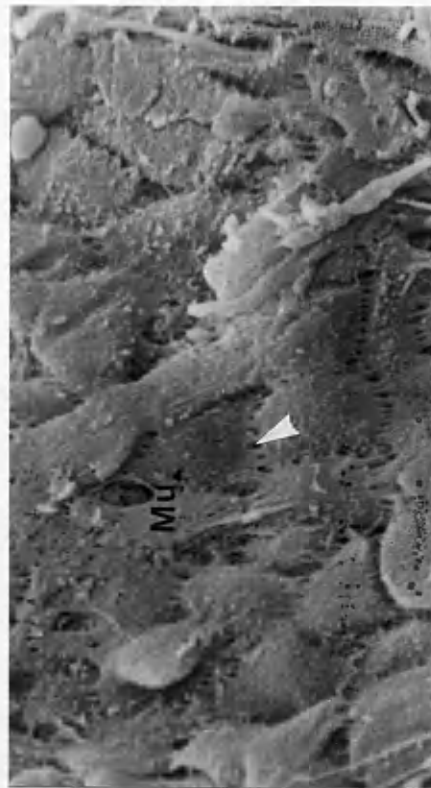
10a



10b



10c



10d

### 3.4 Discussion

The physiological and pharmacological role of  $\text{PGE}_2$  in cervical dilatation has been discussed before in the introduction. A series of ultrastructural changes in the connective tissue (CT) help explain the mechanisms of cervical softening and ripening in late pregnancy and at delivery in sheep (Aughey et al., 1981; Aughey et al., 1983; Parry & Ellwood, 1981) and humans (Danforth, 1960; Minamoto et al., 1987; Yoshida & Manabe, 1990). The connective tissue in the ewes treated with  $\text{PGE}_2$  in the present experiment showed similar changes to those observed by other authors, using LM, TEM and SEM (Aughey et al., 1981; Aughey et al., 1983; Ellwood et al., 1981; Fosang et al., 1984; Owiny et al., 1987) in the ovine cervix at term, when spontaneous ripening takes place. This tissue, in the present study, was characterized by: 1) an increased cellular component with predominance of fibroblasts, 2) a more abundant ground substance and 3) less densely packed bundles of collagen with a reduced density of the fibres of collagen themselves. In contrast, the connective tissue in the control group consisted of a few fibrocytes in a dense matrix of collagen fibres and ground substance. The present findings are similar to those in women (Rath et al., 1987a and 1987b; Uldbejerg, 1981 & 1983; Manabe & Yoshida, 1990) which showed that the histological changes in the connective tissue of the cervix after PG-induced softening both in early pregnancy and at term, mimic those observed with spontaneous dilatation at physiological term.

The pH of the mucus was neutral in both groups ( $\text{PGE}_2$ -treated and controls) in the present study, whereas in late pregnant sheep acidification has been reported

(Aughey et al., 1981; Aughey et al., 1983). This may indicate that the acidification at term is not controlled by  $\text{PGE}_2$ . The pH of the cervical mucus suffers cyclical changes (reviewed by El-Banna & Hafez, 1972) and steroids have been suggested as regulators of these changes. Apparently, the cervical mucus is neutral during the cycle and maximal alkalinity occurs at ovulation, in order to compensate the acidity of the vagina that would otherwise kill spermatozoa. Oestrogens seem to alkalinize mucus. Although in none of the studies were progesterone and oestradiol determined, differences in the oestradiol-progesterone ratio between day 6 of the superovulated cycle in the present study and at mid to late pregnancy (Aughey et al., 1981; Aughey et al., 1983) may explain the absence of acidification in the present study. The 2nd follicular wave of oestrogens that occurs at day 6 (Hauger et al., 1977) may have been responsible for the alkalinity of the cervical mucus in the present study.

Leukocyte infiltration (plasma cells and lymphocytes) in the subepithelial connective tissue was present in the histology of both groups of the present experiment. Leukocyte infiltration in great quantity has been described as another feature of cervical ripening at late pregnancy and intrapartum by most authors, in sheep (Parry & Ellwood, 1981; Fosang et al., 1984; Owiny, 1986), guinea-pigs (Liggins, 1981) and humans (Junqueira et al., 1980; Minamoto et al., 1987). However, in another study (Aughey et al., 1983), it was found in the subepithelial connective tissue of the non pregnant sheep, rather than in the pregnant ones. The absence of great quantity of WBC may be associated with the absence of progesterone withdrawal before labour (Chamley, Buckmaster, Cerini, Cumming, Goding, Obst, Williams and Winfield, 1973) at mid-late pregnancy in Aughey et al.'s work (1983) and at early pregnancy in

the present study, as progesterone is known to be immunosuppressive (reviewed by Liggins, 1981). The SEM micrographs showed presence of WBC, RBC and Platelets at the top of the distorted epithelium of the treated ewes. This is in line with observations of epithelial metaplasia in women (Langley & Crompton, 1973). In the present work therefore, the presence of blood cells in treated ewes was associated with the epithelial metaplasia rather than the connective tissue changes.

In the treated ewes, the usual single columnar epithelium of the cervix (at the internal and mid levels), was gradually substituted by another of multiple layers and polyhedral shaped cells, which resembled the vaginal epithelium. This changes were more obvious with LM, as this technique allows the different strata to be seen. A different view is obtained with SEM, only the top stratum can be seen. SEM micrographs of the treated ewes, were difficult to interpret because of the mixture that existed of mucus and different types of cells at the top of the epithelium. Isolated squames were observed at the internal os and mid cervix in association with white blood cells with SEM, on line with the LM findings. These findings may be interpreted as incomplete metaplasia (Langley & Cropton, 1973), which is the partial replacement of columnar epithelium by squamous epithelium and therefore any mixture of these epithelia may be found. Shed columnar epithelium, cell destruction and spaces left by the columnar cells often containing polymorphonuclears are the features observed at LM that may correspond to the present findings (Langley & Cropton, 1973). Although the LM section were not taken from the specimens of SEM, it appears more likely that the SEM findings described corresponded with the metaplasia observed at LM.

The metaplastic epithelium was observed with Light Microscopy in the crypts and also in the villi of 4/5



treated ewes at the internal os. This is a much higher frequency than in the findings of Lightfoot and Adams (1979), who found that stratified squamous epithelium is present occasionally in crypts of the internal os in control ewes. The finding of this epithelium in both crypts and villi was very uncommon (2/56). The epithelium found in the control ewes of the present work was similarly restricted to the crypts. Another difference when compared with treated ewes was that the columnar cells were maintained at the superficial layer of the stratified epithelium in control ewes. This type of stratified columnar epithelium has been reported as an occasional finding (Cole and Miller, 1935; Raynaud, 1973; Lightfoot and Adams, 1979) in the normal cervix of cyclic ewes.

The stratified squamous epithelium typical of the vagina was commonly observed at the external os of the cervix in the present work, both in the control and treated ewes. This has been reported previously by other authors (Lightfoot and Adams, 1979; Cole & Miller, 1935; Restall, 1965). The finding of this epithelium within the mid cervix would not be very surprising, bearing in mind that this has been found occasionally in the cow (Aughey et al., 1991 personal communication) and also in some cervical crypts in the ewe (Lightfoot & Adams, 1979). The finding of patches of squamous epithelium at mid-cervix, may be explained by the common embryonic origin that some local parts of the cervix may have with the vagina (Mossman, 1973). However the finding of large areas of stratified epithelium different from the stratified columnar at the internal os has not been reported in the normal epithelium of the ewe or the cow. This indicates hyperplasia and certain degree of metaplasia in the columnar epithelium of the treated ewes.

The hyperplastic and metaplastic changes seen in the epithelium, apparently prominent in the treated group,

have not been described before in ultrastructural studies of cervical ripening at term in sheep (Aughey et al., 1983) or after the treatment with PGs in women (Manabe & Yoshida, 1990; Uldbejerg, 1981; Rath et al, 1987a and 1987 b; Theobald et al., 1982). In pregnant sheep which were undergoing changes in the connective tissue at term, the epithelium remained tall columnar (at the int os and mid-cervix) as in the non pregnant ones (Aughey et al., 1983). After PG-induced abortions in women, (Manabe & Yoshida, 1990; Uldbejerg, 1981 & 1983; Rath et al, 1987a and 1987 b), these changes have not been reported, either because they were not present or these authors were only interested in the connective tissue. In woman, epithelial metaplastic changes may occur during infancy and in various stages of pregnancy (Epperson, Hellman, Galvin, and Busby, 1951; Nesbitt, 1955; Krantz, 1973; Langley & Crompton, 1973; Singer, 1976b; Carrow & Green, 1951). Singer (1976b) associates these changes with softening of the cervix and the connective tissue changes that occur during pregnancy. He proposes that as a result of the softening, the cervix everts or gapes and the columnar epithelium is then exposed to the acidic pH of the vagina that induces metaplastic transformation into squamous epithelium.

In the present work, the epithelial metaplastic changes in the cervix were associated with dissociation of collagen fibres in the connective tissue. The ewe treated for less than 8 hours (#6), with very slight softening and collagen dissociation, did not have metaplastic changes. This may also indicate that the exposure of the columnar cells to the low vaginal pH, as a consequence of the softening induced by PGE<sub>2</sub>, may be responsible for the metaplastic changes in the rest of the treated ewes.

Where the human cervix is exposed to the more hostile environment of the vagina (at the external os) it is

lined by thick stratified squamous epithelium identical to that of the vagina. Beneath the region of the squamo-columnar junction, the cervical stroma is often infiltrated with leucocytes, forming part of the defence against the entrance of micro-organisms (Wheather, Burkitt & Daniels, 1987). Likewise in the present study, both the subepithelial infiltration of leucocytes and the epithelial metaplasia may be consequences of the cervical dilatation produced from the vagina by the  $\text{PGE}_2$  and therefore exposure of the inner parts of the cervical tissue to the vaginal environment. The epithelium in the present study was in the luteal phase, when the cervix normally remains closed, and therefore it may be even more vulnerable to the vaginal environment than at oestrus or delivery.

The other alternative is that this metaplasia is induced by the PGE. No metaplastic changes or desquamation of the cervical epithelium have been described before with the administration of PGs. However hormonal-induced metaplasias have been observed with oestrogens ( $\text{E}_2$ ). In the adult sheep, oestrogens can produce metaplastic changes in the cervix. Two different kinds of histological changes are found, depending of the duration of exposure. Short term exposure produces stratified squamous metaplasia of the epithelium (Zuckerman, 1940; Adams, 1977) and in contrast the long exposure to oestrogenic clover or oestradiol, produces persistent changes that histologically resemble the uterus (Adams, 1983; Lightfoot & Adams, 1979). These authors stress the fact that such a metaplasia induced by oestrogens in adult life is unique in the sheep, however oestrogens have been also associated with the induction of hyperplastic and dysplastic changes in cervical epithelium of rodents and primates in adult life (Hellman, Rosenthal, Kistner, and Gordon, 1954; Nesbitt, 1963; Fluhmann, 1954; Ciocca, Puy, Lo Castro, 1986).

The number of animals studied in the present work was small , but there are indications that the use of intravaginal pessaries of PGE2 induce abnormalities in the cervical epithelium, namely proliferation and metaplastic changes. These changes were not observed in the control ewes and therefore do not seem to be associated with the pretreatment with progestagens and PMSG, although the control animals were only three, and therefore the possibility cannot be ruled out completely. PMSG is highly oestrogenic (Evans & Robinson, 1980) and stimulation with oestrogens in turn may produce metaplasia (Zuckerman, 1940; Adams, 1977). In the other hand metaplastic changes and even cancer of the cervical epithelium have been associated with the use of contraceptives in women (Muñoz & Bosch, 1989) but this has not been described in association with the use of progestagen sponges in the ewe (Rainaud, 1973).

These epithelial abnormalities of the cervix are not necessarily pathological in women if they are pregnant, but would be regarded as cancer in nonpregnant adult women (Langley & Crompton, 1973). Cervical cancer in the sheep is rare (McEntee 1990; Thomson, 1988) but is very common in humans (Muñoz and Bosh, 1989). PGE2 is used widely in women to induce abortion or cervical ripening at term. According to the present results in sheep, it would be advisable to investigate whether similar epithelial changes may be induced by PGE2 in humans and if so whether the changes are reversible. The etiology of cervical cancer in woman is unknown, epidemiologists having proposed various risk factors for human cervical cancer that are interdependent, namely: early age at first sexual intercourse, multiple sexual partners and multipary (Muñoz and Bosh, 1989; Brinton, Reeves, Brenes, Herrero, de Britton, Gaitan, Tenorio, García, and Rawls, 1989). The theory claiming that the disease may be venereal is broadly accepted and various workers have proposed human papillomavirus

(HPV) (Muñoz and Bosch, 1989). Previous theories proposed that early age at first sexual intercourse is the key risk factor and that it may be related to a greater vulnerability of the cervix during adolescence (Singer, 1976a). In this cases the sperm has been investigated as a possible mutagen agent (Reid & Coppleson, 1976).

PGE<sub>2</sub> is present in the cervix after coitus from semen (Taylor, 1974), at delivery and when abortion is induced with PGs. The possible PG involvement on squamous metaplasia may be related to various risks factors proposed in cervical cancer. Cervical dysplasia was also more frequent after termination of pregnancy or miscarriage than in nulliparous or after a full time pregnancy (Singer, 1976a). The possibility of PGE<sub>2</sub> involvement in epithelial abnormalities of the human cervix may be worth investigating.

The changes observed in the vaginal connective tissue showed that the local administration of PGE<sub>2</sub> produces the same ultrastructural response in the vagina as in the cervix. Collagenolysis in human vaginal tissue during pregnancy and delivery have been already reported (Manabe & Yoshida, 1986), indicating that vagina also undergoes softening at labour. The present findings indicate that PGE<sub>2</sub> may be involved in this process.

The occasional finding with SEM of secretory epithelium in the caudal vagina of a PGE-treated ewe, may be in line with the findings of Cole & Miller (1935), who reported secretion of mucus by stratified columnar epithelium in the vagina quite early in pregnancy (day 30). The villi of the squamous epithelium in the vagina had "crypts-like" structures in most histological sections from treated ewes, which were similar to those of the columnar epithelium. This may indicate that PGE<sub>2</sub>

induced changes in the vaginal squamous epithelium towards a secretory epithelium.

In the present experiment, differences between the three portions of the cervix were observed. The epithelial and CT changes, in the present experiment, progressed from the vagina to the internal os, the mid-cervix scores being halfway between the internal and external os. These findings are different from those described at term, when no significant difference (Aughey et al., 1983; Fosang et al., 1984) or a different progression (Owiny, 1986; Owiny et al., 1987) were found. Softening was first detected at the internal os in physiological parturition and mid cervix was the last to soften (Owiny, 1986; Owiny et al., 1987). In the other hand, Aughey et al. (1983) and Fosang et al. (1984) did not find any difference in the cervical width, length, water content, consistency, occurrence of the different elements of the CT or the presence of smooth muscle along the length of the cervix of non-pregnant and pregnant sheep at different stages of pregnancy. It is therefore not clear whether these difference exists in the physiological parturition.

The source of PGs at the time of labour seems to be intrauterine tissues, fetal membranes, decidua and myometrium in humans and the sheep (Challis & Olson, 1988), which supports the progressive order of its action from the uterus towards the vagina. On the other hand,  $\text{PGE}_2$  is produced locally within the cervix at term (Ellwood et al., 1981) and that could ensure similar concentrations of uterine PGs along the cervical canal. If the efficacy of the different routes of PG administration in humans is compared, the order is again extra-amniotic, intracervical and finally intravaginal (Calder, 1990). The differences in the microscopical changes along the cervix may be due to the fact that in the present experiment, the exogenous

PGs are progressing in an "antiphysiological direction" from the vaginal pessary towards the uterus and presumably, there was not endogenous PG synthesis by the cervix. All these considerations are important in the use of local administration, to mimic as closely as possible the physiological cervical dilatation at delivery. It will be desirable to either have closer access to the int os of the cervix or to stimulate the local production of PGs by the cervix. Another factor is that most of the action of PGE was probably lost within the vagina, producing unwanted changes in that area and not concentrating at the appropriate target, namely the cervix. It seems therefore that an intracervical route could be more efficient than the intravaginal one, as it has been already suggested for humans (Ulmsten, Ekman, Belfrage, Bygdeman and Nyberg, 1985) and also for sheep (Rickords & White, 1988). Non-surgical embryo collection has been achieved by South African workers (Barry et al., 1990; van Niekerk et al., 1990) in both the sheep and goats using a liquid form of PGE administration that was repeated deeper into the cervix taking advantages of the effects of the first one. Some other factors may have contributed to the successful passage of the cervix, namely the use of general anaesthesia , oestradiol or cervical manipulation with forceps. However, when compared with the present results, they may suggest that the administration of a vaginal pessary followed with an intracervical treatment may reach the cranial parts of the cervix and produce enough dilatation for a complete passage of the cervix.

Unfortunately because of the small number of sheep that was used and the problems with some pessaries being lost , it is not possible to withdraw conclusions about the recommendable administration guide lines of PGE<sub>2</sub>. However, preliminary data from the histology indicates that more marked Connective Tissue changes that reached the int os were observed after more than 16 hours of

the treatment. The probing experiment indicated that there was some action as early as 4 hrs after the treatment, this increased at 24 hrs and the effect was still present after 28 hrs. The results with the 2nd intravaginal pessary were inconsistent, one of the ewes showed the greatest increase of all the groups (passage of 5 cm) whereas in the rest the 2nd pessary made no improvement. The number of ewes was again small, but these results indicated that the 2nd administration may be beneficial if given into the cervix. The results with the use of E2 in the present work are unclear. E2 (administered 4 hrs after the PG) did not enhance the effect of PGE but produced worse results than PGE alone. However E2 on its own seemed to have some positive effect. The number of animals used was very small, but although E2 is believed to have a role in cervical softening at oestrus and parturition (Challis & Olson, 1988), contradicting findings have been already pointed out (Hillier, 1990).

Despite both the dramatic changes seen within the CT at histology and SEM and the softening which was found, the passage of the cervix was impossible. This raises the question of why the connective tissue disaggregation induced by PGE<sub>2</sub> from the vagina was not enough for the cervical passage and what is missing in comparison to the changes that happens at labour. The fact that dilatation was restricted to the caudal part of the cervix has been already discussed. PGE<sub>2</sub> is not the only factor involved in cervical ripening (Hillier, 1990) and steroid are believed to play an important role. The unfavourable oestrogen-progesterone ratio present on day 6 may have contributed to the failure of the cervix to dilate after the treatment with vaginal PGE<sub>2</sub> in the present work. This ratio is characterized at term by progesterone withdrawal and increased levels of oestrogens in the ewe (Chamley et al., 1973), which is believed to promote softening (Challis & Olson, 1988), whereas in humans this does not happen (Liggins,



1983). The pattern in the present experiment was reversed as the sheep were at the luteal phase (day 6 of the cycle) and even more distorted by the superovulatory treatment, producing very high levels of progesterone. However the present findings are in line with a previous study (Rickords & White, 1988) in anoestrous ewes using a similar pessary (Buchanan, Macer, and Yonekura, 1984). Despite the different steroid situation of these two experiments, superovulated ewes in their luteal phase compared with anoestrous ewes (with no CL), the results were similar and slightly better in the present work, suggesting that the high levels of progesterone levels present in the present study did not play a key role undermining the effect of PGE<sub>2</sub>.

Steroid hormones may have effects on cervical ripening but the mechanism and the importance of any of them are not clear (Challis & Olson, 1988; Ledger et al., 1985; Owiny et al., 1987). Direct action, indirect action by promoting the synthesis of PGs or interaction with relaxin have been proposed (Challis & Olson, 1988). It is not clear whether the progesterone withdrawal or the rise in oestrogens play a more important role in cervical ripening. The effect of progesterone, has been stressed by some authors (Liggins, 1983, Fitzpatrick, 1977) and denied by others (Owiny & Fitzpatrick, 1990). The possibility of progesterone suppressing the infiltration of the CT by WBC, which may be a source of collagenase (reviewed by Challis & Olson, 1988 and Calder & Greer, 1990) in the present study has been already discussed.

The oestrogen role promoting softening is claimed because local administration of E<sub>2</sub> induced cervical ripening in woman (reviewed by Steiner & Creasy, 1983) and induced cervical softening at term in the ewe, in the absence of falling progesterone (Owiny et al., 1987). In addition, successful embryo collection trans-

cervically have been reported using local  $\text{PGE}_2$  in combination with intramuscular  $\text{E}_2$  in sheep (Barry et al., 1990) and in goats (van Niekerk et al., 1990). Unfortunately, in the experiments of Barry et al. (1990) and van Niekerk et al. (1990) the role of  $\text{E}_2$  cannot be assessed because of the experimental design, as they did not use  $\text{E}_2$  on its own. Cervical passage for non surgical embryo transfer has been achieved without any drugs with different manipulations of the cervix under general anaesthesia in only 42-59% (Conrood, Coren, McBride, Bowen, and Kraemer, 1986; Kraemer, 1989). In the goat, the cervical folds are less pronounced than in the ewe and the cervix may be passable at oestrus (Evans & Maxwell, 1987) but at day 7, this was almost impossible in controls (van Niekerk, 1990). After the  $\text{PGE}_2$  treatment, the passage was possible without cervical manipulation. The reduced resistance to the passage in this species could explain the higher success of the  $\text{PGE}_2$  treatment compared with the ewe. However the difference between the control and  $\text{PGE}_2$ -treated goats may be explained as in the ewe by the repetition of the treatment further into the cervix or a combination of the factors mentioned. As in the present experiment there are no evidence for the role of the other factors, the thesis maintained is that the intravaginal treatment produced the greatest changes at the external os and vagina, due to the anatomy of this species, and therefore an intracervical treatment may be advantageous.

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